GROWTH DIFFERENTIATION FACTOR 9 SIGNALLING IN THE OVARY

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To Marko and Jesper

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LIST OF ORIGINAL PUBLICATIONS

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I: Bondestam J, Kaivo-oja N, Kallio J, Groome N, Hyden-Granskog C, Fujii M, Moustakas A, Jalanko A, ten Dijke P, Ritvos O. Engagement of activin and bone morphogenetic protein signaling pathway Smad proteins in the induction of inhibin B production in ovarian granulosa cells. Mol Cell Endocrinol. 2002; 195:79-88.

II: Kaivo-Oja N, Bondestam J, Kamarainen M, Koskimies J, Vitt U, Cranfield M, Vuojolainen K, Kallio JP, Olkkonen VM, Hayashi M, Moustakas A, Groome NP, ten Dijke P, Hsueh AJ, Ritvos O. Growth differentiation factor-9 induces Smad2 activation and inhibin B production in cultured human granulosa-luteal cells. J Clin Endocrinol Metab. 2003; 88:755-62.

III: Mazerbourg S, Klein C, Roh J, Kaivo-Oja N, Mottershead DG, Korchynskyi O, Ritvos O, Hsueh AJ. Growth differentiation factor-9 signaling is mediated by the type I receptor, activin receptor-like kinase 5. Mol Endocrinol. 2004; 18:653-65.

IV: Kaivo-Oja N, Mottershead DG, Mazerbourg S, Myllymaa S, Duprat S, Gilchrist RB, Groome NP, Hsueh AJ, Ritvos O. Adenoviral gene transfer allows Smad-responsive gene promoter analyses and delineation of type I receptor usage of transforming growth factor-beta family ligands in cultured human granulosa luteal cells. J Clin Endocrinol Metab. 2005; 90:271-8.

ABBREVIATIONS

aa	Amino Acid
AC	Adenylyl cyclase
Act	Activin
ActRII	Activin type II receptor
Ad-	Adenovirus-
ALK	Activin receptor-like kinase
AMH	Anti-Müllerian hormone
AMHRII	Anti-Müllerian hormone type II receptor
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BAMBI	BMP and activin membrane-bound inhibitor
BMP	Bone morphogenetic protein
BMPRII	Bone morphogenetic protein type II receptor
BRE	BMP responsive element
ca	Constitutively active
cAMP	Cyclic adenosine monophosphate
C-terminal	Carboxy-terminal
CEEF	Cumulus expansion enabling factor
Co-Smad	Common-partner Smad
COX2	Cyclo-oxygenase 2 (Ptgs2)
ER	Endoplasmic reticulum
EGF	Epidermal growth factor
EP2	Prostaglandin E2 receptor
Erk	Extracellular signal-regulated kinase
FS	Follistatin
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
GCNF	Germ cell nuclear factor
GDF	Growth differentiation factor
HAS	Hyaluronan synthase
hGL	Human granulosa-luteal cell
hCG	Human chorionic gonadotropin
I-Smad	Inhibitory Smad
IGF	Insulin-like growth factor
Inh	Inhibin
kd	Kinase defective
KL	Kit ligand
LAP	Latency associated protein
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LTBP	Latent TGF-β binding protein

MAPK	Mitogen activated protein kinase
MH	Mad homology domain
MIS	Müllerian inhibiting substance
m.o.i.	Multiplicity of infection
mRNA	Messenger ribonucleic acid
N-terminal	Amino-terminal
NES	Nuclear export signal
NLS	Nuclear localization signal
OD	Ovarian dysgenesis
OSF	Oocyte secreted factor
PCOS	Poly cystic ovary syndrom
PGC	Primordial germ cell
PGE2	Prostaglandin E2
PKA/B/C	Protein kinase A, B or C
POF	Premature ovarian failure
Ptgs2	Prostaglandin endoperoxide synthase 2 (COX2)
PTX3	Pentraxin 3
PY-domain	Proline-tyrosine rich domain
R-Smad	Receptor-activated Smad
SBE	Smad binding element
SAD	Smad activation domain
SARA	Smad anchor for receptor activation
Ser	Serine
siRNA	Small interfering RNA
Smufr	Smad ubiquitination-related factor
StAR	Steroidogenic acute regulatory protein
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor β
TβRII	Transforming growth factor β type II receptor
Thr	Threonine
TNFIP6	Tumor necrosis factor-induced protein 6
uPA	Urokinase plasminogen activator

ABSTRACT

Since the discovery of the transforming growth factor β (TGF-β) over twenty years ago, more than thirty structurally related proteins have been identified in vertebrates. This family now comprises of activins, inhibins, growth differentiation factors (GDFs), bone morphogenetic proteins (BMPs) and many others. The biological effects of the TGFβ family ligands are mediated to the target cells via heteromeric complexes of type I and II serine/threonine kinase receptors which activate the intracellular Smad signalling protein pathways in various cell types.

In the ovary, two new members of the superfamily were discovered during the 1990s. The oocyte was discovered to express two closely related growth factors that were named growth differentiation factor 9 (GDF-9) and growth differentiation factor 9B (GDF-9B, also known as BMP-15). These proteins are both required for normal ovarian follicle development although their individual significance has been shown to vary between different species. Both GDF-9 and GDF-9B mRNAs are expressed in the human oocytes from the primary follicle stage onwards.

This thesis project was aimed to define the signalling mechanisms utilized by the oocyte secreted GDF-9. We used primary cultures of human granulosa luteal cells (hGL) as our cell model, and recombinant adenovirus-mediated gene transfer in manipulating the TGF-β family signalling cascade molecules in these cells. Overexpression of the constitutively active forms of the seven type I receptors, the activin receptor-like kinases 1-7 (ALK1-7), using recombinant adenoviruses caused a specific activation of either the Smad1 or Smad2 pathway proteins depending on the ALK used. Activation of both Smad1 and Smad2 proteins stimulated the expression of dimeric inhibin B protein in hGL cells.

Treatment with recombinant GDF-9 induced the specific activation of the Smad2 pathway and stimulated the expression of inhibin βB subunit mRNA as well as inhibin B protein secretion in our cell model. Recombinant GDF-9 was also able to activate the Smad3-responsive CAGA-luciferase reported construct and the GDF-9 response in hGL cells was markedly potentiated upon the overexpression of Alk5 by adenoviral gene transduction. Alk5 overexpression also enhanced the GDF-9 induced inhibin B secretion by these cells. Similarly, in a mouse teratocarcinoma cell line P19, GDF-9 could activate the Smad2/3 pathway, and overexpression of ALK5 in COS7 cells rendered them responsive to GDF-9. Furthermore, transfection of rat granulosa cells with small interfering RNA for ALK5 or overexpression of the inhibitory Smad7 resulted in dose-dependent suppression of GDF-9 effects.

In conclusion, this thesis shows that both Smad1 and Smad2 pathways are involved in controlling the regulation of inhibin B secretion. Therefore, in addition to endocrine control of inhibin production by the pituitary gonadotropins, also local paracrine factors within in the ovary, like the oocyte-derived growth factors, may contribute to controlling inhibin secretion. This thesis shows as well that similarly to other TGF-β family ligands,

also GDF-9 signalling is mediated by the canonical type I and type II receptors with serine/ threonine kinase activity, and the intracellular transcription factors, the Smads. Although GDF-9 binds to the BMP type II receptor, its downstream actions are specifically mediated by the type I receptor, ALK5, and the Smad2 and Smad3 proteins.

INTRODUCTION

The most important functions of the ovary are the production of steroid hormones that allow sexual maturation and the production of oocytes capable of fertilization. Several growth factors that belong to the transforming growth factor β (TGF-β) superfamily as well as their receptors and intracellular effector molecules, have been shown to be indispensable for the critical ovarian functions such as oocyte formation and development as well as ovarian folliculogenesis [1-3]. The expression of these signalling cascade molecules is tightly regulated at the different phases of ovarian organogenesis as well as folliculogenesis. Their specific expression patterns determine the responsiveness of the ovarian cell types to each stimulus in the right place and at the right time. Accordingly, flaws along the signalling cascade at either the ligand, receptor or intracellular effector level may cause adverse changes in the intended message leading to fertility defects of varying severity, as has been demonstrated by extensive knockout studies in mice [3].

The TGF-β superfamily comprises of e.g. the three isoforms of TGF-β, the activins and inhibins, growth differentiation factors (GDFs) and bone morphogenetic proteins (BMPs) which all share common sequence elements as well as structural motifs. The superfamily ligands regulate a wide variety of cell functions including proliferation, differentiation, migration as well as apoptosis. They also stimulate the production of extracellular matrix, maintain tissue homeostasis and regulate embryogenesis. The TGF-β superfamily ligands are bioactive as homo- or heterodimers and they signal through a distinct set of type I and II serine/threonine kinase receptors that are located on the plasma membrane. The ligand dimer together with the transmembrane receptors forms a large signalling complex that is capable of activating the intracellular effector molecules, the Smads.

Five type II Ser/Thr kinase receptors, and seven type I receptors have been identified so far. The type II receptor is thought to be constitutively active and it activates the type I receptor by phosphorylating specific serine residues at its glycine-serine rich GS-domain. β-glycan and endoglin function as accessory receptors ("type III receptors") and may modulate the bioactivity of some of the superfamily ligands. When a ligand-receptor complex has formed, different receptor-regulated (R)-Smads are activated through phosphorylation by the type I receptor. Altogether eight different Smads have been found in mammals and they are commonly classified into three groups according to their function. Receptor-regulated Smads (Smad1, -2, -3, -5 and -8) are activated upon ligand binding to the receptor complex, common partner (Co)-Smad (Smad4) translocates into the nucleus with the activated R-Smads and is also required for transcription. Smad2 and Smad3 can be phosphorylated by the activated TGF-β/activin type I receptors, whereas Smad1, -5 and -8 act downstream of the type I BMP receptors. The translocated Smad complexes regulate target gene expression in interaction with other transcription factors, co-activators and co-repressors. The function of the inhibitory (I)-Smads is to block the activation of R-Smads at the type I receptor (Smad7) or their translocation into the nucleus (Smad6). Inhibitory Smads can block TGF-β superfamily signalling by binding to the type I receptors (Smad7)

or by competing with activated R-Smad1 for binding to Co-Smad4 (Smad6). Smad7 can inhibit the activation of both the TGF-β/activin and BMP pathway R-Smads, whereas Smad6 is an inhibitor of BMP signalling (reviewed in [4-6]).

Previously it was thought that the oocyte had a passive role in the events of folliculogenesis and the somatic granulosa cells were the main effectors. However, it is now known that a bi-directional communication axis exists between the oocyte and the surrounding somatic cells; the oocyte regulates the differentiation and proliferation of the granulosa cells which in return secrete factors that control the growth of the oocyte. Consequently, a complex interplay of regulatory factors governs the development of both cell types. Many endocrine, paracrine and autocrine factors including several TGF-β superfamily members are expressed by different cell types in the ovary and they contribute to the regulation and progression of the folliculogenesis.

The first part of this thesis, the review of the literature section, is focused on the TGF-β signalling pathway molecules and signalling mechanisms. In the first part, also the course of ovarian folliculogenesis is shortly reviewed and the biological roles of the oocyte secreted growth differentiation factors GDF-9 and GDF-9B are described. The second part of this thesis is focused on the study of the signalling pathway used by GDF-9.

REVIEW OF THE LITERATURE

1. TGF-β superfamily of growth factors

The first member of the transforming growth factor β (TGF-β) superfamily was discovered in the early 1980s as a growth factor that caused phenotypic transformation and anchorage-independent growth in non-neoplastic rat fibroblast cells [7]. Today, more than thirty structurally related proteins have been identified in vertebrates including the three isoforms of TGF-β, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), activins, inhibins, anti-Müllerian hormone (AMH) and several others. These proteins regulate many of the most important features of living organisms; cell growth, differentiation, migration, apoptosis, embryonic development, morphogenesis and sexual development. Defects along the signalling pathway of the TGF-β superfamily members are associated with a variety of pathological states such as cancer and autoimmune diseases [8].

1.1. Characteristics of the TGF-β superfamily ligands

The different TGF-β superfamily members share certain characteristic structural features and exhibit sequence similarity. All superfamily members are synthesized as large precursor molecules (pre-pro-proteins) consisting of an amino (N-) terminal signal peptide, followed by a pro-region and a carboxy (C-) terminal mature region that is biologically active (Figure 1). The signal peptide directs the protein to the secretory pathway. First, in the lumen of endoplasmic reticulum (ER), the signal sequence is cleaved. Subsequently, the proprotein becomes folded and dimerizes within the ER, and finally proteolytic cleavage in the trans-Golgi network by furin-like endoproteases releases the mature peptide from the pro-region.

Figure 1. TGF-β superfamily ligand structure. *NH_3, amino-terminus; COOH, carboxy-terminus; S, signal sequence; black line, intermolecular disulfide bond; red C, the fourth cysteine residue lacking in GDF-9 and GDF-9B.*

The significance of the pro-region is not well known for each ligand but it has been suggested to be required for proper folding of the mature region [9]. The dimeric pro-regions of the TGF-β isoforms (known as latency associated proteins, LAPs) remain attached to the mature protein via noncovalent associations after cleavage, forming a latent, biologically inactive complex. TGF-β and the associated LAP further form larger latent complexes with latent TGF-β binding proteins (LTBPs). These complexes anchor the latent TGF-β to the extracellular matrix (ECM) due to specific interactions of LTBPs with the ECM components which represents the predominant way of storage of the latent TGF-β. The latent form of TGF-β can be activated through enzymatic or chemical cleavage, although the physiological conditions that promote the activation of TGF-β *in vivo* are not well understood.

One of the major effects of TGF-β is the inhibition of cell proliferation and induction of apoptosis in a variety of cell types [10]. It has recently been shown that the proteolytic activation of latent TGF-β enhances apoptotic response in lung epithelial cells and suppresses both PI-3K/Akt (Phosphoinositide 3 kinase and a serine/threonine kinase also known as protein kinase B, respectively) and p38 MAPK (mitogen activated protein kinase)-dependent survival pathways [11]. While the mature regions of TGF-β dimer form the bioactive molecule, another superfamily member, AMH, instead requires the N-terminal pro-region for optimal bioactivity [12]. The significance of the pro-region for the bioactivity of other superfamily members remains to be further studied.

Another characteristic feature of this protein family is that they contain from six to nine evolutionarily conserved cysteine residues in their C-terminal mature region. Six of these cysteines form three covalent disulfide bonds within the molecule resulting in a tight structure called the cysteine knot [13]. The seventh conserved cysteine residue (fourth consecutive cysteine from the N-terminus) present in most TGF-β superfamily members is involved in forming the covalent intermolecular disulfide bond in the ligand dimer [14, 15]. However, some superfamily members such as GDF-3, GDF-9, GDF-9B/BMP-15 as well as lefty-1 and lefty-2 lack this cysteine residue due to a substitution of an amino acid residue, and therefore these growth factors are unable to form covalent dimers (see Figure 1). Instead, the ligand monomers may remain associated through noncovalent binding [16-20].

2. The signalling pathway

2.1. Type I and II receptors

The members of the TGF-β superfamily are known to signal through specific transmembrane serine/threonine kinase receptor complexes consisting of two type I and two type II receptor molecules. Currently five type II receptors (ActRII, ActRIIB, BMPRII, TβRII and AMHRII) and seven type I receptors are known (activin receptor-like kinase (ALK) 1-7) (see Tables 1 and 2, respectively) [21, 22]. All these receptors are structurally related, although their kinase domains are functionally diverged. The receptor polypeptides consist sequentially of an N-terminal extracellular domain followed by a

transmembrane region and a C-terminal intracellular serine/threonine kinase domain. The type I receptors act downstream of the constitutively active type II receptors and both receptor types are required for proper signalling.

***Table 1**. The type II receptors of the TGF-β superfamily and the ligands known to bind the receptor.*

Type II receptors	Alternative names	Ligands binding to the receptor	References
ActRIIA	ACVR2, ActRII	Activin A, Inhibin A/B, BMP-2, BMP-3, BMP-6/7, BMP-10, GDF-5/6/7, GDF-8 (myostatin), GDF-9B (BMP-15), GDF-11/BMP-11	[23-35]
ActRIIB	ACVR2B	Activin A, Inhibin A/B, Nodal, BMP-2, BMP-6/7, GDF-5, GDF-8 (myostatin), GDF-1, GDF-11	[23, 24, 26-28, 30-33, 36]
AMHRII	MISRII	AMH (MIS)	[37]
BMPRII	BRK3/T-Alk	Inhibin A (with TβRIII), BMP-2/4, BMP-6/7, GDF-5/6, GDF-9, GDF-9B (BMP-15)	[24, 25, 27, 35, 38-42]
TβRII	-	TGF-β	[43]

***Table 2.** The type I receptors of the TGF-β superfamily and the ligands known to bind the receptor.*

Type I receptors	Alternative names	Ligands	References
ALK1	TSR1, SKR3, ACVRL1	TGF-β1, Activin A	[44, 45]
ALK2	ActRIA, TSK7L, SKR1	TGF-β, Activin A, AMH (MIS), BMP-6/7	[23, 24, 33, 40, 45-47]
ALK3	BMPRIA, BMPRI, BRK1, Tfrl1, ACVRLK3	BMP-2/4, BMP-6/7, BMP-10, GDF-5/6/7, AMH	[23, 24, 33, 35, 38, 48, 49]
ALK4	ActRIB, ACVR1B	Activin A, GDF-1 and Nodal (with Cripto), BMP-3, GDF-8, GDF-11/BMP-11	[23, 26, 28-31, 34,36, 45, 50]
ALK5	TβRI, SKR4	TGF-β, GDF-8, GDF-9	[43, 51]
ALK6	BMPRIB, BRK2	BMP-2/4, BMP-6/7, BMP-10, GDF-5/6/7, GDF-9B (BMP-15), AMH	[23-25, 33, 35, 37, 38, 40, 48]
ALK7	-	Nodal	[50]
Accessory receptors			
β-glycan	TGF-β Type III receptor	TGF-β1-3, Inhibin A (with ActRIIA)	[52-54]
Endoglin		TGF-β1,3, Activin A, BMP-2/7	[55, 56]
Cripto		Nodal	[57, 58]
InhBP (Inhibin binding protein)	Inhibin co-receptor	Inhibin A/B (with ALK4)	[59, 60]

The TGF-β superfamily ligands bind differently to their receptors. TGF-β and activin have high affinity to the type II receptor and do not interact with the isolated type I receptor (Massague 1998 [61] and references therein). In contrast, some BMP ligands bind first to type I receptors after which the the type II receptor is recruited to the preassembled receptor-ligand complex [61]. An active ternary complex consisting of a bound ligand dimer and two type I and two type II receptors causes the type II receptor to phosphorylate specific serine residues in the glycine-serine rich region (GS-domain) located upstream of the kinase domain of the type I receptor. The activated type I receptor then further activates the downstream effector molecules of the superfamily, the Smads (see Figure 2 below).

2.2. Accessory receptors

In addition to these twelve classic receptors, other cell surface proteins, such as β-glycan, endoglin and cripto, have also been shown to interact with the TGF-β superfamily ligands and to assist receptor binding. β-glycan was the first such molecule reported and was designated as a type III TGF-β receptor [62], although it is now known to function as a co-receptor for inhibin as well [53]. β-glycan is a highly glycosylated transmembrane protein with a large extracellular domain and a short cytoplasmic tail that lacks kinase activity. It facilitates TGF-β interaction with the type II receptor and promotes inhibin binding to activin type II receptor, antagonizing activin signalling. Endoglin is a homodimeric integral glycoprotein that is expressed widely in vascular endothelial cells. Endoglin cannot bind ligands on its own but facilitates TGF-β1 and -3 binding to their receptors through association with the TGF-β type II receptor [63]. Activin and BMP-7 binding is also facilitated by endoglin through interaction with ActRII and ActRIIB, respectively, and in addition, endoglin can bind BMP-2 via the associated type I receptors [56].

Cripto is a member of the EGF-CFC family of GPI-linked (GPI, a glycosylphosphatidylinositol moiety) membrane anchored proteins and is required for nodal signalling [26, 58]. Cripto interacts directly with ALK4 and nodal, and is required for both the assembly of active ALK4/ActRIIB receptor signalling complexes and Smad2 activation [26]. Interestingly, cripto also antagonizes activin signalling by forming a complex with ActRII and ActRIIB receptors and therefore inhibiting type I receptor recruitment.

A pseudoreceptor called BMP and activin membrane-bound inhibitor (BAMBI) was discovered in *Xenopus* [64] in the late 90s, and based on structural similarities it was later recognized as the same protein as the nma (non-metastatic gene A) gene product identified in a human melanoma cell line [65]. BAMBI is a transmembrane glycoprotein related to the TGF-β superfamily type I receptors, and functions as a negative regulator of TGF-β signalling during *Xenopus* embryonic development [64]. BAMBI lacks the intracellular kinase domain that is required for signalling and it inhibits TGF-β superfamily ligand signalling by forming stable associations with both type I and II receptors in a ligand independent manner [64].

2.3. The Smad protein family

Smad proteins were first identified in the fruit fly *Drosophila melanogaster* in the mid 90s by Sekelsky et al. who found that an intracellular protein named Mad mediates the signalling of decapentaplegic, a member of the TGF-β superfamily corresponding to mammalian BMP-2/4 [66]. The discovery of orthologous proteins in *Caenorhabditis elegans* (Sma-proteins) as well as in vertebrates soon followed, and the newly found protein family was termed Smad [67]. Today, eight different members of the Smad family have been identified in mammals. Based on their function, the Smads are classified to receptor-regulated (R-) Smads (Smad1, -2, -3, -5 and -8), common-partner (Co-) Smads (Smad4 as well as *Xenopus laevis* Smad4α and Smad4β) and inhibitory (I-) Smads (Smad6 and -7). Smads mediate the TGF-β superfamily ligand signalling within the cell functioning as transcription factors that regulate target gene expression.

Depending on the combination of type I and type II receptors and the presence or absence of accessory type III receptors, ligand binding to the receptor complex results in the phosphorylation, and therefore the activation, of the different R-Smads. R-Smad2 and -3 are phosphorylated by activated TGF-β/activin/nodal type I receptors (ALK4,-5 and -7), and Smad1, -5 and -8 act downstream of BMP type I receptors (activated by ALK2, -3 and -6). ALK1 is a TGF-β1 receptor in endothelial cells that activates the Smad1/5/8 pathway. Activated R-Smads form oligomeric complexes with Co-Smad4 and translocate

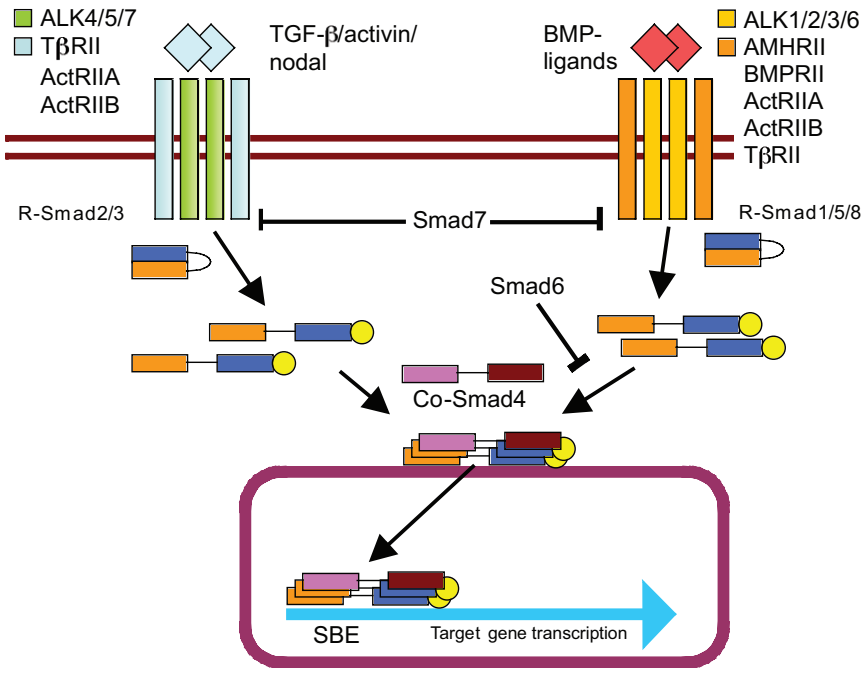

Figure 2. A schematic drawing of the TGF-β superfamily signalling cascade.

into the nucleus where they regulate target gene expression in interaction with a multitude of other transcription factors, co-activators and co-repressors. Inhibitory Smads block TGF-β superfamily signalling by binding to the type I receptors (Smad7) or by competing with activated R-Smad1 for binding to Co-Smad4 (Smad6). Smad7 can inhibit the activation of both the TGF-β/activin and BMP pathway R-Smads, whereas Smad6 is a specific inhibitor of BMP signalling [4-6]. The TGF-β superfamily members signal mainly through two distinct Smad-pathways. TGF-β, activin and nodal can cause the activation of Smad2/3 by the type I receptor, whereas the BMPs activate the Smad1/5/8-pathway (see Figure 2).

2.4. The structure of Smad proteins

Smad proteins contain two conserved domains, an N-terminal Mad homology 1 (MH1) domain and a Mad homology 2 (MH2) domain in their C-terminus, which are connected by a non-conserved proline-rich linker region (see Figure 3). The MH1 domain is highly conserved between the R-Smads and Co-Smads whereas the I-Smads lack a recognizable MH1 domain. In contrast, the MH2 domain is highly conserved among all Smads. The MH1 domain of the R-Smads and Co-Smads binds to DNA with low affinity recognizing specific sequences termed Smad binding elements (SBE) in the promoters of their target genes. Smad2, however, lacks DNA-binding ability due to an insertion in the exon 3 [68]. The MH1 domain confers the ability to interact with other transcription factors and contains a nuclear localization sequence (NLS). In addition, the MH1 domain physically interacts with the MH2 domain exerting an autoinhibitory effect against Smad activation [69].

The linker region connecting the MH1 and MH2 domains is less conserved between the different Smads but contains several important regulatory peptide motifs, including potential phosphorylation sites for MAPKs. A proline-tyrosine (PY) motif present in R-Smads and I-Smads enables Smad interaction with the WW domains of the E3 ubiquitin ligases Smurf1 and Smurf2 (Smad ubiquitination-related factors) [70]. In addition, the Smad4 linker region includes a nuclear export signal (NES) [71] [72] and a Smad activation domain (SAD) that is required in transcriptional complexes mediating the activation of Smad-dependent target genes [73].

The MH2 domain is a multifunctional region that mediates type I receptor recognition, R-Smad oligomerization with Smad4 and transcriptional activation as well as interaction with Smad binding proteins and transcription factors. It is also essential for cytoplasmic anchoring of the R-Smads. R-Smads are activated through phosphorylation of two serine residues at their C-terminal SSXS motif by the activated type I receptor at the inner leaflet of the cell membrane. The I-Smads and the Co-Smads are not phosphorylated at their C-terminus. In addition to the C-terminal phosphorylation site, Smads can be phosphorylated by other kinases as well. The linker region contains phosphorylation sites for extracellular signal-regulated kinase (Erk)-family MAP kinases [74], the Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) [75] and protein kinase C (PKC) [76].

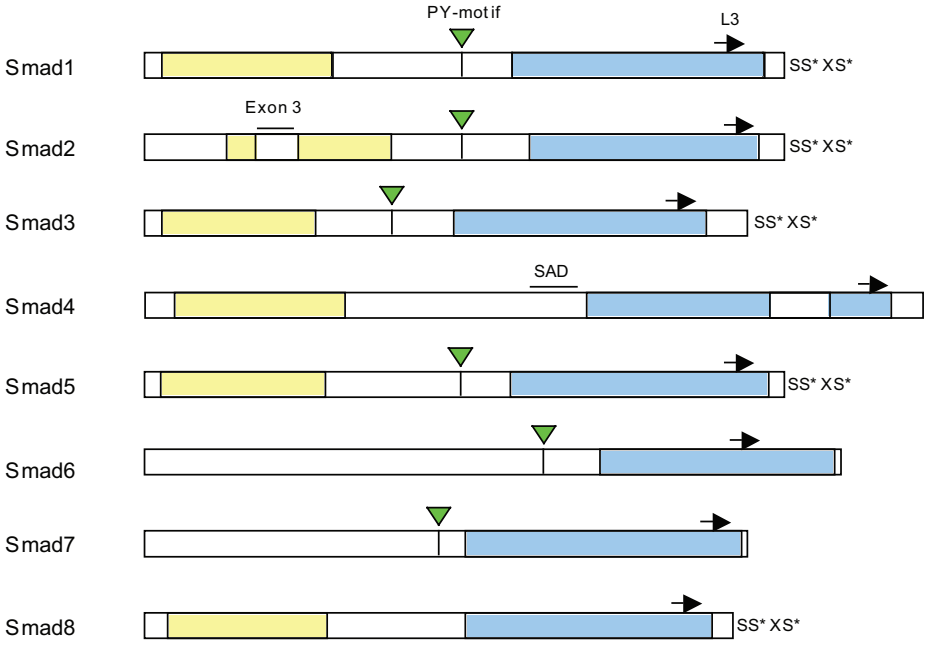

Figure 3. A schematic drawing of the Smad structure. *Yellow colour marks the MH1 domain and blue the MH2 domain. Green triangles show the position of the PY-motif in the linker region and the black arrows denote the L3 loop. Phosphorylated serine residues at the C-terminus of the R-Smads are marked with asterisks. (Modified from Kaivo-oja et al. 2006 [77])*

2.5. Smad-dependent signalling

Inactive R-Smads and Co-Smad4 reside predominantly as monomers in the cytoplasm, whereas the I-Smads localize to the cell nucleus. Smad4 constitutively shuttles between cytoplasm and nucleus and its cytoplasmic localization in unstimulated cells is due to active nuclear export [70, 72]. Cytoskeletal proteins also play a part in the localization and signalling of Smads. Unphosphorylated Smad2 and Smad3 bind microtubule filaments, and their dissociation can be induced by TGF-β treatment [78]. Smads may also interact with filamin, which functions as a scaffold molecule for intracellular signalling proteins that crosslinks actin [79].

Upon ligand binding, the R-Smads are activated by the activated type I receptor at the inner leaflet of the cell membrane through phosphorylation of two serine residues at the C-terminus SS*XS* motif (asterisks highlight the phosphorylated serine residues). Different ligands can induce distinct signalling pathways depending on the composition of the receptor complex. Substrate specificity between the receptor and Smads is determined by the L45 loop in the intracellular domain of the type I receptors, and primarily by the

L3 loop in the MH2 domain in the R-Smads [80, 81]. Upon ligand binding, the activated type I and II receptors are internalized via clathrin coated pits into early endosomes that contain a protein named SARA (Smad anchor for receptor activation) [82]. SARA is a FYVE domain containing scaffolding protein that interacts with the MH2 domain of inactive Smad2 and Smad3 targeting them to early endosomes and aiding the recruitment of Smads to their receptors, thus promoting Smad phosphorylation by TGF-β signalling [83].

A recent report by Runyan et al. shows that endocytosis of the receptor complex is required for proper nuclear translocation of activated Smads, and that internalization enhances the dissociation of phosphorylated Smad2 from the TGF-β-receptor-SARA complex [84]. Internalization of the receptor complex through an alternative route, a lipid raft/caveolar dependent pathway, leads to the degradation of the receptor complex and thus regulates Smad activation and receptor turnover [82]. A cytoplasmic protein named PML (promyelocytic leukaemia tumour suppressor) physically interacts with Smad2/Smad3 and SARA and is required for the association of Smad2 and -3 with SARA. Its expression is induced by TGF-β and it is required for the accumulation of SARA and TGF-β receptors in the early endosomes [85]. Several other accessory/scaffolding proteins like SARA have been discovered for the TGF-β/activin/nodal pathway R-Smads e.g. axin and disabled2 (Dab2) [86, 87], but none are yet known for the BMP-pathway Smads.

Following phosphorylation, Smads can form oligomers at different stoichiometries; heterotrimers consisting of two R-Smads and one Smad4 or heterodimers. Activated Smads accumulate into the nucleus where they control target gene expression in a cell type specific manner through interactions with other transcription factors, corepressors and coactivators. Diverse ligand responses in different cell types are the result of different Smad-interacting transcription factors and of cooperation with other signalling pathways.

2.6. Smad-independent signalling and alternative Smad phosphorylation

In addition to the canonical Smad signalling pathway, other signalling pathway molecules have been found to participate in mediating the physiological responses to the TGF-β superfamily ligands. For example, TGF-β is known to activate Erk, JNK and p38 MAPK kinase pathways (reviewed in [88]), although the exact mechanism and biological responses are not well characterized. These parallel signalling pathways may co-operate with the Smad pathway to elicit specific biological responses. The non-Smad signalling molecules may also directly modify Smad function through alternative phosphorylation, and Smads may modulate the activity of other signalling proteins as well. For example, TGF-β-induced expression of human matrix metalloproteinase 13 (MMP-13) gene in gingival fibroblasts is dependent on the activation of two distinct signaling pathways, Smad3 and p38 MAPK [89].

Members of the MAP kinase family are frequently involved in TGF-β/Smad signalling, and Ras activated Erk kinases can modulate the nuclear accumulation of the activated Smads. Epidermal growth factor (EGF), hepatocyte growth factor as well as oncogenic Ras stimulate Erk kinases, which in turn phosphorylate Smad proteins. Erk kinase

phosphorylates specific serine residues in the linker regions of Smad1 [74], Smad2 and Smad3 [90].

Phosphorylation of the Smads may also result from the activation of MAPK/Erk kinase kinase 1 (MEKK1), which acts downstream from Ras and upstream from growth factor-induced Erk MAPK and stress-activated SAPK/JNK pathways [91]. Phosphorylation of the Smads in the MAP kinase sites in the linker region attenuates ligand-induced nuclear translocation and alters Smad-dependent transcription. Activation of Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) also results in Smad2, Smad3 and Smad4 phosphorylation and inhibits TGF-β-induced nuclear import and transcriptional activity of Smad2, and affects Smad heteromerization [75]. Protein kinase C (PKC) abrogates the DNA binding ability of Smad3 [76]. Also Smad6 and Smad7 can be phosphorylated independently of TGF-β stimulation [92].

3. Regulation of TGF-β superfamily signalling

TGF-β superfamily signalling is regulated at multiple levels. Ligand access to the receptors can be regulated by extracellular antagonists (ligand traps) and specific BMP-binding proteins, or other TGF-β family ligands. At the cell membrane level the signal-transduction pathway may be broken by the pseudoreceptor BAMBI that can compete with the type I receptors for ligand binding and inhibit TGF-β, activin and BMP signalling [64, 93]. Receptor internalization provides another point for the modulation of the signal transduction as discussed above. Intracellular regulation may take place through the actions of inhibitory Smads and the regulation of Smad expression levels as discussed above.

3.1. Extracellular antagonists and binding proteins

Several extracellular antagonists that inhibit TGF-β superfamily ligand signalling have been identified such as noggin, chordin/SOG (*Drosophila* short gastrulation protein), chordin-like, DAN/Cerberus-protein family (including Dan, Cerberus, Gremlin, Dante and protein related to Dan and Cerberus PRDC), sclerostin, follistatin and follistatin related protein (FSRP). Many of these antagonists form high affinity complexes selectively with the BMPs and inhibit their biological functions. They play an important role during the *Xenopus* embryonic development in establishing a proper BMP-morphogen gradient [94]. For example, Noggin, that was first identified as a regulator of the dorsal development in *Xenopus* embryos [95], is an inhibitory binding protein for BMP-2, -4, and -7 as well as GDF-5 [96-99]. Chordin also binds BMP-2, -4 and -7 and antagonizes their signalling through preventing their binding to the BMP receptors [100]. Proteins belonging to the DAN/Cerberus family bind the BMPs similarly to noggin and chordin. Follistatin binds activin with very high affinity and BMP ligands (BMP-2, -4, -7 and -15/GDF-9B) as well as GDF-11 with lower affinity [23, 101-104]. Follistatin binds activin preventing its binding to the receptor, and binds the BMP receptors through the BMPs,

thus forming a trimeric complex [102]. Inhibin is a member of the TGF-β superfamily and a naturally occurring antagonist of activin. Inhibin antagonizes activin action in the pituitary gonadotropes through binding to the activin receptors ActRIIA and ActRIIB thereby blocking activin binding [105].

3.2. Intracellular modulators

The basal levels of Smad proteins are regulated post-translationally through a ubiquitin-mediated proteasomal degradation pathway. Both the size of the Smad pool in unstimulated cells and the levels of activated Smads are thus regulated. E3 ligases, Smurf1 and -2 as well as SCF/Roc1 antagonize TGF-β family signalling through interaction with R-Smads, targeting them for degradation and terminating Smad-mediated signalling [106-108]. Therefore, Smurf-mediated degradation regulates R-Smad levels and the sensitivity of cells to incoming signals.

In contrast to the R-Smads, expression of the inhibitory Smad6 or Smad7 is highly regulated by extracellular signals. Induction of Smad6 and Smad7 expression by BMP and TGF-β represents an auto-inhibitory feedback mechanism for ligand-induced signalling [6, 109]. Recruitment of a complex of Smad7 with either Smurf1 or Smurf2 to the type I TGF-β receptors at the cell membrane results in receptor ubiquitination and degradation. Internalization of the receptor complex bound to Smad7 via lipid raft caveolin-positive compartments promotes poly-ubiquitination and results in accelerated receptor turnover [82]. Activation of the EGF receptor and possibly other tyrosine kinase receptors, interferon-γ signalling through STAT (signal transducer and activator of transcription) proteins, and activation of NF-κB by tumour-necrosis factor-α (TNFα), also induce Smad7 expression [88].

In addition to the Smurfs, inhibitory Smad6 and Smad7 also modulate the Smad-mediated signalling. Smad7 inhibits both TGF-β and BMP pathway Smad activation through interaction with the type I receptor, whereas Smad6 blocks only BMP pathway Smads by competing with the activated R-Smads for heteromeric complex formation with Co-Smad4 [110]. The co-repressors c-Ski and SnoN are two highly conserved members of the Ski family of proto-oncoproteins and they can antagonize TGF-β signalling through direct interactions with Smad4 and the R-Smads inhibiting transcription of target genes [111, 112].

Dephosphorylation of the Smads by specific phosphatases is a mechanism for the termination of Smad signalling. PPM1A/PP2Cα was recently found to function as a Smad phosphatase and to play a critical role in terminating TGF-β signalling through dephosphorylation of Smad2 and Smad3 [113]. Therefore, phosphorylation of the Smads not only causes their activation but also modulates their activity and provides a mechanism for integration of the Smad pathway with other signalling pathways that can modulate TGF-β superfamily signalling.

4. Folliculogenesis and TGF-β superfamily members in ovarian function

The principal function of the female gonad is the production of the mature oocyte for fertilization and proliferation. Therefore, the establishment of the germ line that gives rise to the oogonia and ultimately the oocytes is of fundamental importance for animal reproduction. It was long thought that the oocyte plays a passive role during folliculogenesis, but over the past decade or so, this view has changed dramatically. The oocyte is now known to secrete factors that regulate the differentiation and growth of the surrounding somatic cells, promoting follicle growth. In response, the somatic cells secrete factors that modulate oocyte development. One example of this bi-directional communication is the oocyte secreted factor-Kit ligand regulatory loop. The immature oocyte produces factors that stimulate Kit ligand secretion in granulosa cells [114]. Kit ligand binds to the c-kit receptors on the oocyte and promotes oocyte growth [115]. As the oocyte reaches full size, the stimulatory effect turns to inhibition of Kit ligand synthesis [114]. Therefore, the oocyte modulation of granulosa cell function is developmentally regulated.

4.1. Ovarian organogenesis

In mice ovarian organogenesis begins when the extraembryonic ectoderm induces germ cell determination [116]. During the early embryonic development, uncommitted epiblast cells in the extra-embryonic mesoderm involute through the primitive streak to the yolk sac endoderm and become committed as primordial germ cell (PGCs) [117]. These cells proliferate and migrate via the yolk sac into the hind gut endoderm and dorsal mesentery, and finally to the genital ridges during gastrulation. As they reach their destination, PGCs lose their motility, become encapsulated by the primary sex cords and differentiate depending on the available sex chromosomes into oogonia or spermatogonia [118]. The cortical sex cords give rise to the female ovaries, [119] whereas the medulla slowly breaks down (reviewed in [77]).

The process of gametogenesis starts as the PGCs leave the dorsal mesentery and continues as they enter and colonize the genital ridges to establish the prospective gonad. Gametogenesis involves the transition of PGCs into oogonia, which undergo further DNA replication until they finally enter meiosis to become oocytes. In the developing gonads, the oogonia become surrounded by mesonephros derived somatic cells and form cortical cords or clusters of germ cells. Individual primordial follicles, where the oocyte is surrounded by a unilayer of squamous (pre)granulosa cells and a basal lamina, emerge from the distal ends of the cortical cords concomitantly with the initiation of meiosis (reviewed in [77, 118-120]).

In humans, the ovaries start to form during the first weeks of gestation, and oogonia appear around the 6th to 7th week of gestation. The oogonia first divide mitotically and colonize the primitive ovary. Oogonia become oocytes as they enter meiosis, and by the 20th week of gestation the number of oocytes is at the greatest (6-7 million). The pool of oocytes slowly diminishes, and at birth only 300 000-400 000 oocytes remain enclosed

within follicles [121, 122]. At puberty, only two hundred thousand primordial follicles are left [123]. Of these, only about 400 are ovulated during reproductive life while the rest undergo atresia. By menopause, which today occurs in most women at the age of ~51, the ovarian reserve of primordial follicles is depleted.

4.2. Overview of ovarian folliculogenesis

4.2.1. Gonadotropin-independent growth

The pool of primordial follicles within the ovaries remains dormant until the onset of puberty, although some follicles are continuously recruited to the growth phase. As primordial follicles undergo transition into primary stage, the size of the oocyte increases and the somatic pregranulosa cells around the oocyte become cuboidal and are thereafter termed granulosa cells [118, 120]. When two or more layers of granulosa cells have developed, the follicles are called secondary or preantral follicles. Granulosa cells proliferate very slowly at the early stages of folliculogenesis: in humans the transition from the primary to the secondary follicle stage may take as long as 120 days [124]. At the secondary stage, the oocyte grows extensively, the granulosa cells become more proliferative and a theca cell layer is recruited from interstitial stromal cells to surround the follicle outside the basal lamina. The presence of the oocyte in preantral follicles inhibits granulosa cell progesterone synthesis and premature luteinization [125]. Multilayered follicles are poorly vascularized, since the basal lamina inhibits the intrusion of blood vessels from thecal layers to the granulosa cell layer.

Oocyte growth is required to achieve meiotic competence and for the oocyte to be able to resume nuclear maturation needed for fertilization and cell cleavage. During the growth phase, the oocyte also differentiates and secretes a glycoprotein rich membrane, the zona pellucida, that coats the oocyte. Granulosa cells become coupled with each other as well as with the oocyte via gap junctions passing through the newly formed zona pellucida [126]. The gap junctions facilitate the bi-directional communication between the oocyte and granulosa cells and allow the transfer of small molecules.

As folliculogenesis proceeds, a fluid filled antrum is formed within the multilayered granulosa cells. Antrum formation divides the granulosa cells into two distinct compartments, the cumulus granulosa cells closely surrounding the oocyte and the mural granulosa cells lining the follicle wall within the basal lamina (see Figure 4). Folliculogenesis may proceed from primordial follicles through primary and secondary stages into early antral stage independently of the gonadotropins, despite the fact that granulosa cells gain responsiveness to FSH already at the secondary follicle stage in humans when they begin to express FSH receptors [127].

4.2.2. Gonadotropin-dependent growth

The default pathway of an ovarian follicle is to undergo atresia at the early antral stage, but as levels of circulating FSH rise at puberty, a few antral follicles are rescued from atresia by FSH and are recruited to continue growth during each menstrual cycle. One of the growing follicles becomes chosen as the dominant follicle that develops into a mature Graafian follicle competent of fertilization while the rest undergo atresia. At the antral stage, the theca cells differentiate into theca externa and theca interna. They begin to express LH receptors and produce androgens under the influence of LH. Granulosa cells of selected follicles continue to express FSH receptors and begin to express LH receptors (FSHR and LHR, respectively) as well as P450 aromatase, gaining the ability to produce estrogen from thecal androgens. Among the recruited follicles the leading follicle grows faster and produces higher levels of estrogens and inhibins than the rest. The expression of follistatin, an activin antagonist, is also upregulated during the antral phase and causes a shift in the activin/inhibin balance towards inhibin. Together, estrogens and inhibins produced by the largest follicle suppress pituitary FSH production and as a result the remaining selected follicles are deprived of adequate FSH stimulation required for survival. After antrum formation the two granulosa cell compartments differentiate. Mural granulosa cells continue to express LH receptors and produce progesterone at low levels, while the cumulus cells surrounding the oocyte have a low steroidogenic capacity and a high proliferative activity. In addition, the oocyte secretes factors that suppress LHR expression in the cumulus cells. At the antral stage the immature oocyte has completed its growth and is competent to resume meiosis (reviewed in [119, 124]).

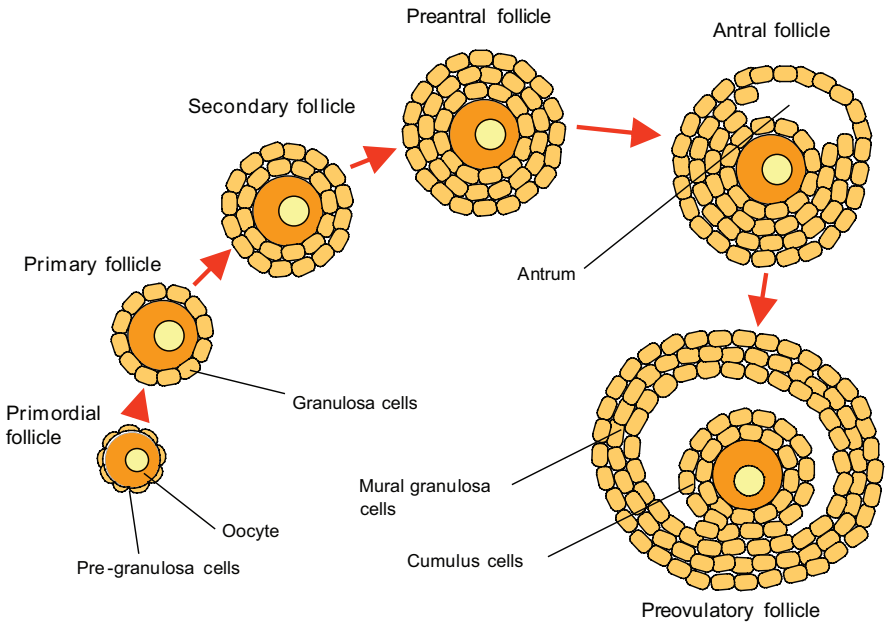

Figure 4. A schematic drawing of the stages of folliculogenesis

4.2.3. Ovulation and corpus luteum formation

It has been estimated that the transition from the primary to the secondary follicle stage takes more than 120 days in humans. In addition, the secondary follicle develops into an antral follicle in 71 days and from this stage the development to a preovulatory stage takes a further 14 days [124]. The last seven days before ovulation are marked with an increased growth of the dominant follicle. The LH surge triggers the onset of the ovulation process and within ~36 hours the follicle wall ruptures and the oocyte is released into the peritoneal cavity. During the ovulation process, the gap junctions between the oocyte and granulosa cells are disrupted and the oocyte undergoes a series of changes in its nucleus as well as in the cytoplasm, known as oocyte maturation. Oocyte meiosis is resumed and arrested again at Meiosis II until fertilization.

The LH surge also shifts the granulosa steroid production from estrogen to progesterone and induces the cumulus granulosa cells to secrete a hyaluronic acid rich extracellular matrix in a process called cumulus expansion. This matrix is important for oocyte extrusion from the follicle and for ovum pick-up by the oviduct fimbria. Biochemically, the ovulation process resembles an acute inflammation, as eicosanoids, prostaglandins and proteolytic enzymes are upregulated and play a role in rupturing the follicle wall.

The remaining follicle degenerates into a corpus luteum after ovulation. Vasculature and fibroblasts from the surrounding theca cell layers penetrate the basal lamina and the mural granulosa cells begin the uptake of cholesterol and luteinize. The main function of the corpus luteum is to produce progesterone under the influence of LH. The corpus luteum maintains steroid production for 14±2 days after which it declines into avascular corpus albicans if pregnancy does not begin.

4.3. TGF-β superfamily ligands in folliculogenesis

Within the ovary, the progress of folliculogenesis is in part regulated by peripheral endocrine factors, the pituitary gonadotropins FSH and LH as well as growth hormone (GH) and prolactin in some species. In addition, intraovarian factors, such as steroids, cytokines and other growth factors act in a paracrine/autocrine manner and co-ordinately contribute to the processes of recruitment, development, atresia, selection and ovulation of follicles. The growth of the follicle is considered to be gonadotropin-independent to the small antral stage and during these early phases folliculogenesis is considered to be driven by these local autocrine and paracrine signals from the oocyte and the surrounding somatic cells [77].

A complex bi-directional communication axis between the oocyte and granulosa cells as well as between the granulosa and thecal cells drives the progression of follicle development at these early stages. Five different classes of growth factors, including insulin-like growth factor (IGF), TGF-β, TGF-α, fibroblast growth factor (FGF) and cytokines have been described within human ovarian follicles.

The role of TGF-β superfamily ligands in ovarian organogenesis as well as folliculogenesis has been studied extensively in animals. From these studies it is known

that various TGF-β superfamily members are expressed by the oocyte, granulosa and theca cells in a developmental-stage related manner. Among the local factors at least activins, inhibins, TGF-βs, BMPs, growth differentiation factor 9 (GDF-9) and GDF-9B (also known as BMP-15) as well as AMH (also known as Müllerian inhibiting substance, MIS) are required during the development of follicles [119]. The developing oocyte is known to express BMP-6, GDF-9 and its close homolog GDF-9B [17, 18, 128]. Recently, Dragovic et al. discovered that oocytes from preovulatory mouse follicles also express inhibin βA and βB subunit mRNAs, although the secretion of the respective proteins remains to be further studied [129]. The granulosa cells produce at least activins, inhibins, TGF-βs, BMP-2, BMP-3 and BMP-6 as well as AMH at different stages of folliculogenesis, while the theca cells can produce all the isoforms of TGF-β, BMP-3b, BMP-4 and BMP-7 [130, 131] (reviewed in [77]).

For appropriate signalling, an intact signalling cascade from ligands and receptors to intracellular effectors and accessory proteins has to be present and functional. The temporal and spatial regulation of these signalling cascade molecules determines the responsiveness of the cell type to each stimulus and the direction of signalling within the follicle is dependent on cellular distribution of the whole signalling pathway. Of the TGF-β superfamily ligands particularly the BMPs together with their antagonists have been shown to play a prominent role throughout embryonic development and organogenesis. Gene ablation studies in mice have identified BMP4, -8b and -2 as regulators of primordial germ cell (PGC) formation from the epiblast cells [2]. Targeted mutations of either BMP-4 or BMP-8b lead to severe defects in PGC formation in the embryos that survive gastrulation [2]. In addition, reproductive defects have been found in knockout mice for different ovary expressed TGF-β superfamily signalling proteins, such as activin βB, GDF-9B/BMP-15, GDF-9, AMH, ALK6, AMHRII, inhibin α and Smad3 [2, 3] (reviewed in [77]).

5. Oocyte-secreted growth differentiation factors 9 and 9B

5.1. GDF-9 gene and protein structure

GDF-9 was first discovered in 1993 in search for new TGF-β related sequences from mouse genomic DNA [16]. The mouse GDF-9 gene is encoded by two exons separated by a 2.9 kb intron and codes for a 1.7 kb messenger RNA (mRNA) [132]. The GDF-9 gene localizes to the autosomal chromosome 11 in the mouse (11 B1.3; 11 29.0 cM) and chromosome 5 in the human (5q23-5q33.1). The predicted amino acid sequence of mouse GDF-9 shows a predicted signal peptide of 29 amino acids in the N-terminus (determined by SignalP 3.0 Server; http://www.cbs.dtu.dk/services/SignalP), followed by a 277 aa pro-region that contains a tetra-basic proteolytic processing site at position 303-306, and a 135 aa C-terminal mature region that shows significant sequence similarity with known TGF-β superfamily members [16]. The mouse GDF-9 pro-peptide contains four potential N-glycosylation sites at asparagines 163, 229, 258 and 325. GDF-9 lacks the fourth out of seven conserved cysteine residues in the C-terminal region [16] that is required for the

formation of a covalent dimer, a characteristic of most TGF-β superfamily ligands [14, 15]. Therefore, GDF-9 may only form dimers through noncovalent interactions.

In most mammalian species, GDF-9 transcripts and protein are expressed specifically in the growing oocytes from the primary follicle stage onwards until fertilization [133-138]. In bovine and ovine ovaries, GDF-9 transcripts can, however, be detected already in primordial follicles [139]. Fitzpatrick et al. have reported GDF-9 mRNA expression also in non-ovarian tissues such as testis and hypothalamus [140], but its function in these organs remains unclear.

5.2. Transcriptional regulation of GDF-9

Recently, regulatory elements that confer high-level expression of GDF-9 in the oocyte were reported by Yan et al. [141]. The proximal ~400 base pairs upstream of the GDF-9 gene in several mammalian species contain a conserved E-box sequence (CAGCTG) that enables oocyte-specific expression of GDF-9 [141]. E-box sequences have been identified in several tissue-specific gene promoters, and they are involved in tissue specific protein expression through binding basic helix-loop-helix (bHLH) transcription factors [142, 143]. Figla, also known as Fig-α, (Factor in the germline alpha) is a germ cell specific transcription factor that binds to the E-box motif present e.g. in the promoter of oocyte specific zona pellucida genes together with another transcription factor E12, and regulates the expression of ZP1-3 proteins [144]. As of yet, it is not known, however, which factor or factors regulate the onset of GDF-9 expression in the oocyte.

Germ cell nuclear factor (GCNF) is a member of the nuclear receptor superfamily that functions as a transcription factor to repress target gene transcription. Both the GCNF mRNA and protein are expressed in the oocyte at the primary, secondary and pre-ovulatory stages but not in primordial follicles in the adult mouse [145]. GCNF recognizes a direct repeat of an AGGTCA-sequence with zero base pair spacing (DR0) [146] and can bind to the DR0 elements on the GDF-9 promoter repressing GDF-9 gene transcription [147]. In oocyte specific GCNF knockout mice ($GCNF^{fl/fl}Zp3Cre$), GDF-9 as well as its homologue GDF-9B are up-regulated during diestrus due to lack of GCNF repression [147].

5.3. GDF-9B gene and protein structure

A close homolog of GDF-9 was discovered five years after the discovery of GDF-9 simultaneously by two research groups using homology-based cloning approaches. This growth factor was named GDF-9B (or BMP-15), and it is also expressed in the oocyte from the primary follicle stage continuing through ovulation [17, 18]. The gene encoding for mouse GDF-9B maps adjacent to the centromere on the X-chromosome, and to Xp11.2 in the human X-chromosome [18, 133]. The mouse GDF-9B gene consists of two exons separated by a 4.2 kb intron, coding for a 392 aa pre-pro-peptide consisting of a 25 aa N-terminal signal peptide (predicted by SignalP 3.0 Server; http://www.cbs.dtu.

dk/services/SignalP), followed by a 242 aa pro-domain and a C-terminal 125 aa mature domain. The biologically active mature protein is cleaved from the pro-domain at the tetrabasic proteolytic cleavage site (residues 264-267) during protein processing. There are five potential N-glycosylation sites in the mouse GDF-9B protein located at asparagines 85, 213, 236, 349 and 373. GDF-9B, like GDF-9, contains only six out of the seven conserved cysteines present in the mature domain of most TGF-β superfamily ligands, and also in GDF-9B the missing cysteine residue is replaced by serine residue [17, 18]. In addition, the mature mouse protein contains two additional cysteine residues upstream from the first conserved cysteine like the TGF-βs and activins.

6. Biological functions of GDF-9 and GDF-9B

6.1. GDF-9 Knockout mouse

Soon after the discovery of GDF-9, a knockout mouse model was created to define its roles in mammalian development. The knockout model showed that GDF-9 is essential for normal ovarian folliculogenesis as the knockout mice display arrested follicular development at the primary follicle stage and are completely infertile while heterozygous females and male knockout mice are not affected [134]. The transition of primordial follicles to primary follicles is not impaired in GDF-9 deficient mice, however, suggesting that GDF-9 may not be required for the onset of growth.

GDF-9 deficient mice exhibit severe defects in folliculogenesis; the oocyte differentiation is compromised and the oocytes grow more rapidly compared to control oocytes whereas the follicle growth ceases at the primary stage [148]. Although the oocytes can achieve full size, their meiotic competence acquisition is impaired and they present several ultrastructural abnormalities. The granulosa cells surrounding the oocyte do not proliferate, but also fail to undergo apoptosis [134]. The theca cell layer fails to form, which is confirmed by the absence of several theca cell markers [149]. In addition, the knockout mice have elevated serum FSH and LH levels [134]. Molecular characterization of the defects in GDF-9 deficient mouse ovarian follicles has revealed that the primary follicles demonstrate an up-regulation of inhibin α and Kit ligand, suggesting that GDF-9 inhibits Kit ligand expression in granulosa cells in a paracrine manner [149]. Interestingly, in mice lacking both GDF-9 and inhibin α, folliculogenesis progresses further than the primary follicle stages, and follicles with multiple granulosa cell layers can be found before the mice develop ovarian tumours (late preantral stage). This suggests that the granulosa cells may proliferate even without the presence of GDF-9 and that up-regulation of inhibin α prevents the granulosa cell proliferation in GDF-9 knockout mice [150]. In addition, the follicles of GDF-9/inhibin α knockout mice become surrounded by a theca cell like layer although no theca cell markers can be found, indicating that theca cell recruitment can occur but their differentiation is blocked. Also their inhibin βA and βB subunit as well as Kit ligand expression is up-regulated [150].

6.2. GDF-9 functions in pre-antral follicles

Vitt et al. have shown that intraperitoneal (ip) injection of recombinant GDF-9 *in vivo* stimulates primordial and primary follicle progression in mice and increases the expression of CYP17 (cytochrome $P450_{17}\alpha$ hydroxylase/lyase), a theca cell marker in ovarian homogenate [151]. CYP17 is an enzyme involved in the steroidogenesis of thecal androgens (see Figure 5). Both IGF-1- and FSH-induced estradiol and progesterone production are suppressed by recombinant rat GDF-9 in small and large bovine granulosa cells [152]. Injection of porcine GDF-9 gene fragments into prepubertal gilts caused an increase in the number of primary, secondary and tertiary follicles concomitantly with the decrease in the number of primordial follicles [153]. In the hamster, GDF-9 has been shown to enhance primordial and primary follicle formation [154]. In contrast, Nilsson and Skinner were unable to show stimulation of the progression of primordial follicles *in vitro* with recombinant GDF-9 but showed that it promoted the early primary follicle progression in the rat [155].

In human, recombinant rat GDF-9 promotes the growth, development and survival of primordial ovarian follicles up to the secondary follicle stage in organ culture [156], and in the cow, GDF-9 stimulates the proliferation of granulosa cells from both small and large follicles in the presence of IGF-I and FSH [152]. Treatment with recombinant rat GDF-9 either alone or in the presence of FSH stimulates the growth of preantral follicles isolated from immature rats as measured by the follicle diameter [135]. In organ culture, GDF-9 has been shown to promote human ovarian follicle growth, development and survival within thin slices of ovarian cortical tissue [156]. In neonatal rat ovary explants, treatment with rat recombinant GDF-9 also enhances inhibin α protein content, a differentiation marker for early follicles [135]. Recombinant GDF-9 suppresses the differently spliced Kit ligand (KL) transcripts KL-1 (principally existing in soluble form) and KL-2 (mainly membrane bound) expression in mouse preantral follicles [157].

Very recently, it was discovered that GDF-9 prevents rat granulosa cell apoptosis during preantral to early antral stage by inhibiting the activation of PI-3K/Akt pathway [158]. Down-regulation of GDF-9 by morpholino oligonucleotides induced follicle cell apoptosis causing an increase in caspase-3 activity and suppressed preantral follicle growth *in vitro* [158]. GDF-9 also protected granulosa cells from ceramide-induced apoptosis in early antral follicles but not in late antral or preovulatory follicles. In addition, the presence of GDF-9 was required to maintain the FSH receptor mRNA expression during preantral to early antral transition [158].

6.3. GDF-9 functions in antral follicles

Recombinant rat GDF-9 functions as a granulosa cell mitogen of early antral as well as preovulatory rat follicles. It also modulates granulosa cell steroidogenesis by suppressing FSH stimulated progesterone and estradiol production [159] but stimulates basal steroidogenesis in the absence of FSH [149, 159]. In primary theca cells and immortalized theca-interstitial cells, GDF-9 stimulated both basal and LH-induced

androstenedione synthesis *in vitro* [160]. In addition, recombinant GDF-9 suppresses FSH-induced LH receptor expression in both antral and preovulatory follicles and FSH-induced cAMP synthesis in small antral follicles whereas in preovulatory follicles the effect is smaller [159]. Figure 5 illustrates the steroid synthesis pathways in theca and granulosa cells. In human granulosa cells, GDF-9 also inhibits cAMP-induced steroidogenesis in preovulatory human granulosa cells [161]. Recombinant GDF-9 suppresses both KL-1 and KL-2 mRNA expression in mouse mural granulosa cells from antral follicles without affecting the KL-1:KL-2 ratio [157].

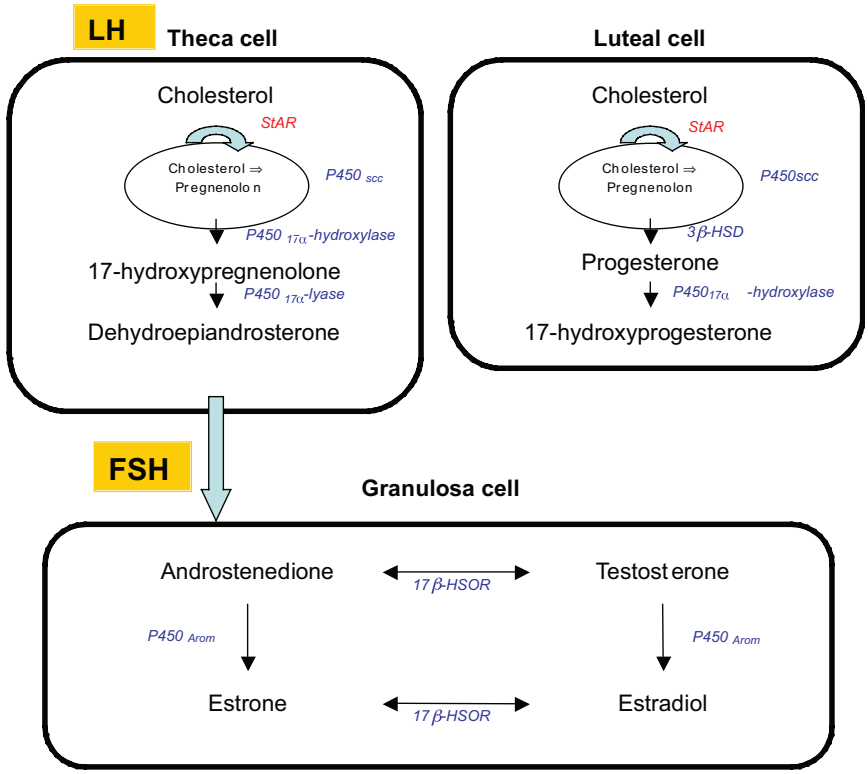

Figure 5. Progesterone and estrogen synthesis pathways. *StAR regulates the transport of cholesterol into the inner membrane of the mitochondrion, which is the rate limiting step in the steroid synthesis pathway. LH regulates the cholesterol transport into the mitochondrion. $P450_{scc}$ catalyses the cholesterol side chain cleavage within the mitochondrion while the rest of the steroid synthesis pathway enzymes are located in the smooth endoplasmic reticulum. FSH stimulates the aromatization ($P450_{aromatase}$ action) of thecal androgens into estrogens in granulosa cells. (StAR, steroidogenic acute regulatory protein; $P450_{scc}$, side chain cleavage; 3β-HSD, hydroxy steroid dehydrogenase; $P450_{arom}$, aromatase; HSOR, hydroxy steroid oxido-reductase).*

Oocytes are known to secrete factors that promote follicle growth, and GDF-9 can partly mimic this oocyte effect [162]. A GDF-9 neutralizing antibody mAb-GDF-9-53 can completely block the GDF-9-induced granulosa proliferation whereas it can only partially block the mitogenic activity of denuded oocytes cultured with mural granulosa cells [162].

6.4. GDF-9 during cumulus expansion

Recombinant mouse GDF-9 has been shown to modulate the expression of some proteins involved in cumulus expansion therefore mimicking the actions of the oocyte. GDF-9 can induce hyaluronan synthase 2 (HAS2), cyclo-oxygenase 2 (COX-2, prostaglandin endoperoxide synthase 2) and steroidogenic acute regulatory protein (StAR) mRNA synthesis and suppresses urokinase plasminogen activator (uPA) and LHR mRNA expression in granulosa cells from antral follicles in mice [138]. In addition, recombinant mouse GDF-9 can induce cumulus expansion in oocytectomized cumulus-oocyte complexes (COCs) in the absence of oocytes [138]. HAS2 is expressed in mouse cumulus cell-oocyte complexes after gonadotropin surge and just preceding efficient cumulus expansion and is involved in the synthesis of the hyaluronic acid rich matrix by the cumulus granulosa cells during cumulus expansion [163]. Inhibition of uPA, a serine protease that cleaves plasminogen into its active proteolytic form plasmin, prevents the degradation of this matrix [164]. StAR regulates the transport of cholesterol from the outer membrane of the mitochondrion to the inner membrane during progesterone synthesis in granulosa cells, whereas COX-2 catalyzes the rate limiting first step in the synthesis of prostaglandins (especially prostaglandin E2, PGE_2), which may be involved in the induction of ovulation, cumulus expansion and implantation [165-167]. GDF-9 also enhances prostaglandin E_2 receptor (EP2) mRNA expression in preovulatory mouse granulosa cells, and can stimulate progesterone synthesis through a PGE_2–EP2 pathway [168]. Down-regulation of LH receptor mRNA expression in the granulosa cells inhibits their responsiveness to LH and as a result, luteinization.

Recently, it was reported that injection of GDF-9 double stranded RNA (RNA interference) can block the fully grown mouse oocyte GDF-9 mRNA, but not the GDF-9B mRNA expression, and also reduce the GDF-9 protein levels [169]. In addition, cumulus cells incubated with GDF-9 dsRNA-injected oocytes showed only limited cumulus expansion compared to control cumulus cells cocultured with buffer-injected and dsGDF-9B-injected oocytes. HAS2 and Ptgs2 (prostaglandin-endoperoxide synthase 2, COX-2) mRNA levels were downregulated in cumulus cells cocultured with GDF-9 dsRNA, suggesting that GDF-9 could be the oocyte secreted factor enabling cumulus expansion [169]. However, in another study, the specific GDF-9 neutralizing antibody mAb-GDF-9-53 could not completely neutralize the oocyte-induced cumulus expansion of oocytectomized cumulus-oocyte complexes (OOX), although it could neutralize the recombinant GDF-9-induced expansion of the OOX complexes [170]. Also, oocyte induced HAS2 expression could not be blocked completely by the antibody, while it could attenuate GDF-9-induced HAS2 transcription. Finally, the soluble form of the extracellular

domain of BMPRII, the type II receptor for GDF-9 in granulosa cells [42], antagonized GDF-9-induced expansion but only partially antagonized the oocyte-induced expansion [170]. Thus, the results from Dragovic et al. suggest, that GDF-9 cannot alone account for the actions of the oocyte secreted cumulus expansion enabling factor (CEEF).

6.5. GDF-9 target genes

Only a few direct target genes for GDF-9 have been identified so far in the ovary. GDF-9 has been shown to induce HAS2, COX-2 as well as StAR mRNA expression and to suppress uPa and LHR mRNA expression in cultured mouse granulosa cells [138]. With a microarray approach, Varani et al. found that GDF-9 induces pentraxin 3 expression in mural granulosa cells of preovulatory mouse follicles and tumour necrosis factor-induced protein 6 (TNFIP6) in all granulosa cells after the LH surge [171]. Pentraxin is a secretory protein that belongs to the long pentraxin family of inflammatory proteins. It is expressed by the cumulus cells during cumulus expansion and localizes into the matrix [172]. Recombinant mouse GDF-9 also induces peroxiredoxin 6 expression in bovine cumulus cells [173]. Prostaglandin E_2 (PGE_2) as well as prostaglandin E2 receptor (EP2) expression is also promoted by GDF-9 administration [168]. Recently, Pangas et al. identified gremlin, a BMP antagonist, as a gene regulated by GDF-9 in mouse granulosa cells from large antral follicles [174]. However, gremlin does not inhibit GDF-9 activity although it can suppress the BMP-4-induced prostaglandin E_2 production [174].

7. GDF-9B in the ovary

7.1. GDF-9B deficient mice

GDF-9B-deficient male mice are normal and fertile, whereas female mice exhibit reduced fertility due to defects in the ovulation process and the ability of oocytes to develop into normal embryos [175]. A superovulation protocol with the GDF-9B$^{-/-}$ mice revealed that some oocytes were trapped within the follicle and were not ovulated. In addition, the trapped oocytes were denuded and larger than normal, and were only surrounded by a few cumulus cells [175]. Double mutant mice lacking both alleles of GDF-9B and one allele of GDF-9 have more severe fertility defects than the GDF-9B knockout. These mice display abnormalities in folliculogenesis, cumulus cell function and fertilization [175].

7.2. GDF-9B functions in the ovary

Like GDF-9, recombinant GDF-9B functions as a granulosa cell mitogen, promoting rat granulosa cell proliferation independent of FSH effects [176]. GDF-9B suppresses FSH-induced progesterone synthesis while having no effect on estradiol production or basal steroidogenesis [176]. GDF-9B inhibits FSH-dependent granulosa cell differentiation by

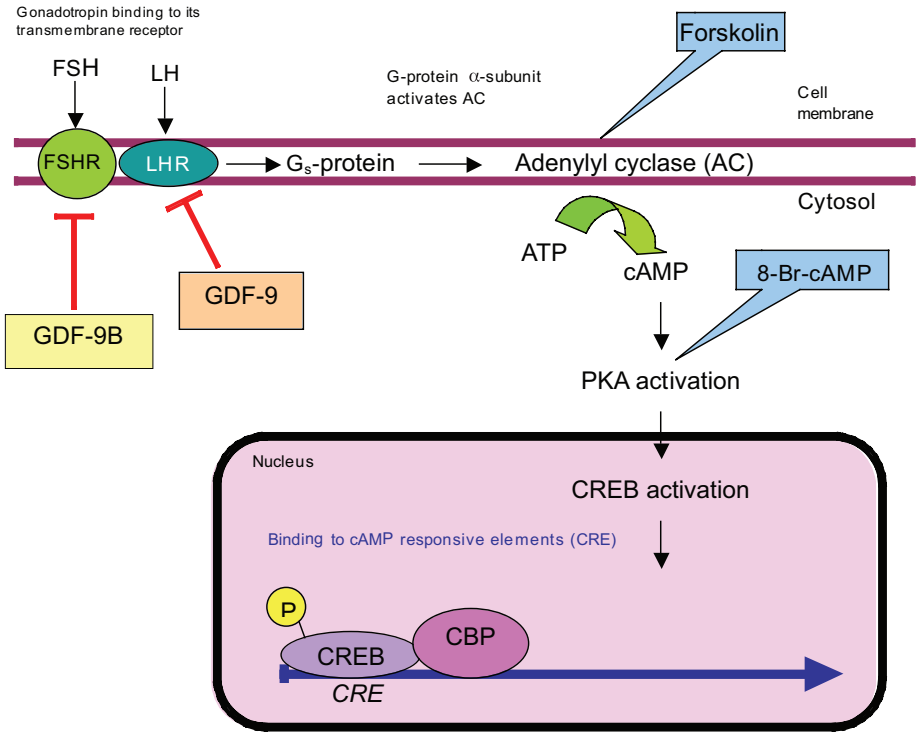

Figure 6. A schematic drawing of the gonadotropin signalling pathway. *Gonadotropin binding to its transmembrane receptor activates a stimulatory G-protein. The $G_s\alpha$-subunit activates adenylyl cyclase (AC) that catalyses the conversion of ATP into cAMP. Forskolin is a diterpene and an activator of adenylyl cyclase. The second messenger cAMP activates protein kinase A (PKA) which can also be activated by a stable 8-Br-cAMP molecule. PKA causes the phosphorylation, and therefore the activation or inactivation, of downstream effector proteins that regulate target gene expression. CREB is a cAMP responsive element binding protein (CREB) that functions as transcription factor and CBP is a protein binding CREB. GDF-9 inhibits the expression of the LH receptor in granulosa cells while GDF-9B can block the expression of the FSH receptor mRNA.*

suppressing the expression of the FSH receptor mRNA, leading to the suppression of FSH-induced increases of StAR, $P450_{scc}$, $P450_{arom}$, 3β-hydroxysteroid dehydrogenase, LHR and inhibin subunit mRNA expression in rat granulosa cells [176]. FSH binding to its transmembrane receptor induces the activation of a stimulatory G-protein which in turn activates adenylyl cyclase (AC) (see Figure 6 for a schematic of the gonadotropin signalling pathway). AC catalyses the synthesis of a second messenger cyclic AMP from ATP, and activation of intracellular protein kinase A (PKA) pathway by cAMP causes the phosphorylation of downstream proteins eventually leading to regulation of target gene transcription. GDF-9B acts upstream of the cAMP signal since GDF-9B could not inhibit forskolin-induced increase of the above mentioned proteins (Figure 6) [176]. Follistatin, an inhibitor of activins [101], BMP-2, -4 [102] and -7 [23], can antagonize the GDF-9B suppression of FSH receptor expression and attenuate GDF-9B induced granulosa cell proliferation by binding directly to the GDF-9B molecule [177].

GDF-9B also stimulates FSH synthesis and secretion in primary culture of rat pituitary gonadotrope cells without affecting LH and gonadotropin releasing hormone receptor (GnRH-R) expression [178]. Both GDF-9 and GDF-9B modulate the expression of Kit ligand (KL) in granulosa cells, GDF-9 inhibiting and GDF-9B promoting its expression [179]. Kit ligand expression in granulosa cells suppresses the oocyte GDF-9B mRNA synthesis, establishing a negative paracrine feedback loop within the follicle [179]. Blocking of c-Kit, the oocyte expressed receptor of KL, by a c-kit antibody suppressed the GDF-9B-induced granulosa cell proliferation, suggesting that the involvement of c-kit signalling is important for GDF-9B stimulated granulosa cell mitosis [179]. Oocytes can prevent cumulus cell apoptosis in bovine COCs, and the administration of GDF-9B, BMP-6 or -7 to oocytectomized complexes reduced cumulus cell apoptosis significantly, whereas GDF-9 had no effect [180]. However, follistatin, that antagonizes GDF-9B action [104], or BMP-6 neutralizing antibody could only block approximately 50% of the oocyte-induced anti-apoptotic effect [180].

Recently, Yoshino et al. studied the expression pattern of the C-terminal mature domain of GDF-9B in mouse ovaries and discovered that the mature form of the protein is barely detectable in the oocytes of preovulatory follicles but its expression is significantly increased in the oocytes just before ovulation [181]. In addition, GDF-9B was shown to induce cumulus expansion and the expression of EGF-like growth factors as well as some molecules downstream the EGF signalling pathway in the cumulus cells, including COX2, HAS2, pentraxin 3 and tumour necrosis factor-induced protein 6 [181]. Interestingly, all of these genes have also been found to be upregulated by GDF-9 [138, 171]. Gueripel et al. also reported recently that GDF-9B expression is increased during gonadotropin-induced follicular development and is associated with cumulus expansion in immature mice [182]. These results are in line with the findings in GDF-9B knockout mice that have defects in the ovulation process, suggesting that GDF-9B plays a major role in the cumulus expansion in the mouse. Table 3 below compares the effects of GDF-9 and GDF-9B during the different stages of folliculogenesis.

	GDF-9 effects	GDF-9B effects
Preantral follicles	granulosa cell mitogen	
	primary follicle progression	
	suppression of Kit ligand expression	promotes Kit ligand expression
	prevents GC apoptosis in the rat	
Antral and pre-ovulatory follicles	granulosa cell mitogen	granulosa cell mitogen
	modulation of steroidogenesis,	suppression of FSH-induced progesterone production, no effect on estradiol production
	suppression of LHR expression	suppression of FSHR
	induction of pentraxin 3 and gremlin expression	
Cumulus expansion	induction of cumulus expansion	induction of cumulus expansion
	induction of HAS2, COX2, StAR, PGE2, EP2	prevents cumulus cell apoptosis
	suppression of uPA, LHR	induction of EGF-like growth factors

Table 3 Comparison of GDF-9 and GDF-9B effects in the granulosa cells.

8. Naturally occurring mutations in GDF-9 and GDF-9B genes

8.1. Human mutations

In 2004, a rare deletion mutation in the human GDF-9 gene was described in heterozygous sisters with spontaneous dizygotic twins [183]. A four base pair deletion beginning at position 207 from the start of exon 1 alters the open reading frame and introduces a premature stop codon leading to the truncation of the GDF-9 protein in the pro-region and therefore a loss of function. Eight single nucleotide polymorphisms (SNPs) were discovered from screening the coding region of GDF-9 in a vast sample of mothers with dizygotic twins. However, no evidence could be found that these common variations within the human GDF-9 locus contributed to the frequency of dizygotic twinning [183]. However, recently Palmer et al. found six rare novel variants in GDF-9 in mothers of dizygotic twins [184]. The novel variants are significantly more common in mothers of dizygotic twins suggesting that rare GDF-9 variants may contribute to the likelihood of dizygotic twinning [184].

Screening of women with premature ovarian failure (POF) has also revealed mutations in the GDF-9 gene [185, 186]. POF manifests itself in malfunction of the whole ovary, and is a cause of female infertility. Its symptoms include loss of functional follicles in women under 40 years of age, amenorrhea for at least 6 months and high gonadotropin levels and low estrogen levels [187]. Two missense mutations have been found in Indian women (substitution of lysine for glutamate, K67E; and of valine for methionine, V216M) and one in a patient of Caucasian origin (substitution of serine for tyrosine, S186Y), which were associated with POF [185, 186]. All of these mutations were found in the pro-region of the protein. Takebayashi et al. failed to identify mutations in human GDF-9 or -9B genes in POF and polycystic ovary syndrome patients (PCOS) but this may be due to a limited sample size used in the study (15 patients with POF and 38 patients with PCOS) [188].

Mutations in the human GDF-9B gene have also been identified in patients with POF. The first human mutation was described in sisters with hypergonadotropic ovarian failure due to ovarian dysgenesis (OD) [189]. A heterozygous non-conservative substitution in the second exon causes the substitution of a tyrosine residue for a cysteine within the GDF-9B pro-region (Y235C). The mutant protein seemed to be processed abnormally, which may be caused by the cysteine substitution that could affect the protein dimerization [189]. Recently, several missense substitutions in the human GDF-9B gene have been found in POF-patients as well, suggesting that the GDF-9B gene is highly associated with the etiology of ovarian failure [190, 191]. All of the described mutations are located across the pro-region of GDF-9B, as in the case of GDF-9. In six cases the substitution involves an arginine residue, but the significance of this is currently unknown (see Table 4 for a summary of naturally occurring human mutations in both GDF-9 and GDF-9B).

	DNA sequence variation	Amino acid substitutions	Location within protein	Phenotype	References
GDF-9	199 A > C	K67E	Pro-region	POF, SA	[185]
	Deletion of base pairs 207-210 in exon 1		Pro-region	Dizygotic twinning	[183]
	307 C>T	P103S	Pro-region	Dizygotic twinning	[184]
	362C>T	T121L	Pro-region	Dizygotic twinning	[184]
	392-393 insT			Dizygotic twinning	[184]
	557 C > A	S186Y	Pro-region	POF, SA	[186]
	646 G > A	V216M	Pro-region	POF	[185]
	1121C>T	P374L	Mature-region	Dizygotic twinning	
	1268-1269 del AA			Dizygotic twinning	[184]
	1360C>T	R454C	Mature-region	Dizygotic twinning	[184]
GDF-9B	181 C > T	R61W	Pro-region	POF	[191]
	182 G > A	R61E	Pro-region	POF	[191]
	202 C > T	R68W	Pro-region	POF	[190]
	226 C > T	R76C	Pro-region	POF, PA	[191]
	227 G > A	R76H	Pro-region	POF	[191]
	443 T > C	L148P	Pro-region	POF	[186]
	538 G > A	A180T	Pro-region	POF, PA	[190]
	538G>T + 539C>T	A180F/S+V	Pro-region	POF	[191]
	588 T > A	N196K	Pro-region	POF	[191]
	617 G > A	R206H	Pro-region	POF	[191]
	631 C > T	E211X	Pro-region	POF (homozygous)	[191]
	661 T > C	W221R	Pro-region	POF	[191]
	704 A > G	Y235C	Pro-region	Ovarian dysgenesis	[189]
	727 A > G	I243G	Pro-region	POF	[191]

Table 4. Naturally occurring mutations in human GDF-9 and GDF-9B genes possibly affecting the ovarian phenotype. *(POF, premature ovarian failure; SA, secondary amenorrhea; PA, primary amenorrhea).*

8.2. Sheep mutations

A naturally occurring mutation in the GDF-9 gene was recently discovered in the Belclare and Cambridge sheep strains. The mutation causes sterility and primary ovarian failure in homozygous ewes due to abnormal follicle development but an increased ovulation rate and fertility in heterozygotes compared to wild-type sheep [192]. This mutation (named $FecG^{H}$) changes an uncharged polar serine residue in the GDF-9 mature domain into a nonpolar phenylalanine located in a region that is predicted to be involved in type I receptor binding, possibly affecting the ability of the mutated GDF-9 to bind to its receptor [192]. Some antral follicles have been found in the mutant ewe ovaries but the oocytes in this stage follicles are often degenerated, and also the arrangement of the granulosa and cumulus cells are irregular and abnormal [193].

Five different mutations in the GDF-9B gene have been discovered in sheep that affect fertility and ovulation rate. All these mutations cause an arrest in folliculogenesis at the primary follicle stage in homozygous animals resembling the phenotype of the GDF-9 knockout mouse. In contrast, heterozygous ewes for the individual GDF-9B mutations exhibit increased ovulation rates and fertility. Most GDF-9B mutations are located in the mature region of the protein, but the $FecX^{G}$ mutation causes a premature stop codon in the proregion and therefore no bioactive mature protein can be produced. Table 5 below lists the known mutations in the sheep GDF-9 and GDF-9B genes that affect fertility and ovulation rate.

There is a clear discrepancy between the phenotypes of the homozygous GDF-9B knockout mice that are only subfertile and the completely infertile GDF-9B mutant ewes but the reason for this difference is not fully understood. It has been suggested however, that the differences may derive from the low- vs. poly-ovulatory nature of these species, or the different relative importance of these growth factors in sheep and mice [175, 194]. In contrast, a mutation in the human GDF-9B gene located in the proregion was found recently in two sisters with hypergonadotropic ovarian failure [189]. The patients had streak ovaries resembling the homozygous GDF-9B mutant sheep ovaries.

The effect of gene dosage of GDF-9 and GDF-9B can be mimicked by immunoneutralizing the GDF-9 and GDF-9B proteins. Short term immunization of sheep against either GDF-9 or GDF-9B using a DEAE dextran adjuvant (low efficacy immunization) can lead to an increased ovulation rate having no apparent harmful effects on fertilization of the released oocytes or subsequent pregnancy [195]. In contrast, long term immunization with an adjuvant causing a strong immune response (i.e. Freunds adjuvant) can result in abnormal follicular development and anovulation [196]. In sheep, both GDF-9 and GDF-9B have been shown to be important for the follicular development unlike in mice, and in addition, ovulation rate in sheep is dependent on the changes in the dosage of either of these factors.

Gene	Allele	Base change	Coding residue (aa)	Mature peptide residue (aa)	Amino-acid change	Reference
BMP-15	$FecX^G$	C → T	239	(Pro-region)	Gln → STOP	[192]
	$FecX^B$	G → T	367	99	Ser → Ile	[192]
	$FecX^I$	T → A	299	31	Val → Asp	[197]
	$FecX^H$	C → T	291	23	Glu → STOP	[197]
	$FecX^L$	G → A	321	53	Cys → Tyr	[198-200]
GDF-9	$FecG^H$	C → T	395	77	Ser → Phe	[192]

Table 5. Naturally occurring mutations in sheep GDF-9 and GDF-9B genes. *The sheep strains with GDF-9B mutations: $FecX^G$ and $FecX^B$ alleles are found in Belclare Cambridge strains; $FecX^I$ Inverdale; $FecX^H$ Hanna; $FecX^L$ Lacaune. GDF-9 mutation $FecG^H$ is found in Cambridge and Belclare sheep strains.*

9. GDF-9 and GDF-9B protein processing

Liao et al. have demonstrated that human GDF-9 and GDF-9B can readily form noncovalently linked homodimers when expressed individually and heterodimers when co-expressed [201]. The proproteins of the co-expressed GDF-9 and GDF-9B in the heterodimer, however, seem to be less susceptible to proteolytic cleavage, since less mature GDF-9/GDF-9B protein is secreted compared to singly expressed proteins [201]. Introduction of the sheep $FecX^I$ (substitution of valine residue at position 31 for aspartate, V31D) or $FecX^B$ (substitution of serine residue 99 for isoleucine, S99I) mutation into human GDF-9B protein weakens the processing efficiency of the proprotein compared to wild type when expressed alone, and severely reduces or abolishes the processing and secretion of the mutant GDF-9B when co-expressed together with human GDF-9 [201, 202]. Also, the processing of the co-expressed GDF-9 proprotein into mature protein is simultaneously impaired [201]. When human GDF-9 protein carrying the sheep mutation $FecG^H$ (substitution of serine residue 77 for phenylalanine, S77F) was co-expressed with mutant GDF-9B, the secretion of both proteins was significantly reduced [202]. An interesting observation was made by Hashimoto et al. that interactions between the proregion and the mature protein of mouse GDF-9B seem to prevent the proper processing of the proprotein [203] as no mature protein is produced. The mouse proregion is also able to reduce significantly the production of human mature domain in a chimeric construct [203].

AIMS OF THE STUDY

The aims of this thesis project were to uncover the signalling components of the oocyte growth factor GDF-9. We wanted to study whether GDF-9 can signal through the canonical TGF-β superfamily pathway or whether it utilizes some other receptor-intracellular mediator molecules.

Specific aims were:

- To develop a cell model for studying the TGF-β superfamily signalling components in ovarian context (I)
- To study the effect of GDF-9 on inhibin B production in human granulosa cells (II)
- To determine whether either the Smad2/3 or the Smad1/5/8 pathway is activated by GDF-9 (II)
- To determine the type I and II receptors involved in the signalling of GDF-9 (III and IV)

MATERIALS AND METHODS

Please refer to the individual studies (I-IV) for more detailed protocols.

1. Human granulosa-luteal cells

Human granulosa luteal cells used in these studies were obtained by follicular aspiration from patients undergoing infertility treatments in Finnish infertility clinics Family Federation of Finland, Felicitas IVF Clinic and Department of Obstetrics and Gynaecology University of Helsinki. The ovaries were stimulated by administrating a human GnRH analog and human recombinant gonadotropin. Thirty-six to thirty-seven hours prior to oocyte retrieval the patients were administered a total dose of 10 000 IU of hCG. The granulosa cells from 1-6 patients aspirated the same morning were pooled, enzymatically dispersed with 1% hyaluronidase in Dulbecco's Modified Eagle's Medium at 37°C for 30 min. Separation of the granulosa-luteal cells from erythrocytes was achieved by centrifugation through Ficoll-Paque. After this the cells were either recovered for RNA extraction or plated on plastic culture dishes at densities specified in studies I-IV and cultured in DMEM supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin at 37°C in a 95% air – 5% CO_2 humidified environment.

2. Rat granulosa cells

Granulosa cells were obtained from small antral follicles of estrogen-treated rats. Ovaries were punctured in L-15 Leibovitz medium. Ovarian debris, oocytes, and small follicles were removed, and the remaining medium containing granulosa cells was collected after low-speed centrifugation at 500 × g for 10 min. Granulosa cells were resuspended into the culture medium (McCoy's 5a supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin).

3. Cell lines

Human embryonic kidney 293T cells and COS7 cells (originating from African green monkey kidney fibroblast-like cells) were grown in DMEM whereas P19 cells (derived from mouse embryonic carcinoma cells) were cultured in α-MEM supplemented with 10% FCS, 2mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin at 37°C in a 95% air - 5% CO_2 humidified environment in standard 175 ml plastic cell culture flasks and passaged when the cells reached 60-70% confluency.

4. Animals

Immature female rats (Sprague-Dawley, 25 d old, body weight from 50–60 g) were obtained from Charles River Laboratories (Wilmington, MA). Animals were anesthetized and killed using CO_2 72 h after insertion of diethylstilbestrol implants. All animals were housed under controlled humidity, temperature, and light regimen and fed *ad libitum* on a standard rat chow. Animal care was consistent with institutional and National Institutes of Health guidelines.

5. Expression of mouse and rat recombinant GDF-9

Tagged and untagged recombinant rat GDF-9 protein has been previously described [135]. A cell line expressing fully processed mouse GDF-9 was also developed and used as a source of bioactive recombinant GDF-9 protein. The mouse full-length cDNA [17] was subcloned into pEFIRES-P expression vector [204] and transfected into HEK 293T cell line by FuGene 6 (Roche Diagnostics) transfection reagent. Cells expressing high levels of the recombinant protein were selected with increasing concentrations of puromycin in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. The recombinant protein was produced into serum-free harvesting medium (DMEM/Ham's F-12, 1:1) supplemented with L-glutamine and 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B, 0,01% (vol/vol) BSA and 100 µg/ml heparin. Levels of recombinant protein were estimated in immunoblots using the purified N-tagged rat GDF-9 as a standard [135].

6. Generation of monoclonal antibodies against GDF-9

A synthetic peptide to annotated amino acids 420-450 of the C-terminal part of the human GDF-9 of sequence VPAKYSPLSVLTIEPDGSIAYKEYEDMIATKC (GenBank accession no. NP 005251) was made using F-moc chemistry, coupled to tuberculin through the cysteine thiol using a heterobifunctional agent, and used to immunize mice by standard methods. After an initial immunization and two boosts at monthly intervals, the sera of the mice collected by tail bleed were tested against ELISA wells coated directly with the immunizing peptide. High responding mice were boosted iv, and 4 days later the spleens were removed and used for fusion to SP2/0 splenocytes by standard methods. Positive hybridomas were tested in Western blot against recombinant GDF-9. Monoclonal antibody 37 was purified by protein A chromatography using a high salt protocol. The antibody has minimal cross-reaction with preparations of recombinant GDF-9B/BMP-15.

7. Expression plasmids and reporter gene constructs

The pGL3(CAGA)$_{12}$-luciferase reporter plasmid was provided by Dr. C. H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden) [205]. The pGL3(BRE)-luciferase reporter plasmid [206] and the expression plasmid pcDNA3-Smad3 were provided by Dr. P. ten Dijke (The Netherlands Cancer Institute, Amsterdam, The Netherlands). pcI-ALK4 was provided by Dr. A. Klibanski (Massachusetts General Hospital, Boston, MA) and subcloned in the pcDNA3 vector (Invitrogen). The pcDNA3-ALK1, pcDNA3-ALK2, pcDNA3-ALK3, pcDNA3-ALK5, pcDNA3-ALK6, pcDNA3-ALK7, pcDNA3-Smad3, pcDNA3-Smad6, and pcDNA3-Smad7 expression plasmids were from Dr. P. ten Dijke (The Netherlands Cancer Institute, Amsterdam, The Netherlands. The reporter pGL2 (GCCG)$_{15}$-lux was provided by Dr. K. Kusanagi (Japanese Foundation for Cancer Research, Tokyo, Japan[207].

8. Recombinant adenovirus constructs

High titer stocks of the recombinant Ad-GFP, Ad-(CAGA)$_9$-luciferase, hemagglutin (HA)-tagged wild-type Ad-ALK5 and Ad-ALK4, FLAG-tagged Ad-Smad1/2/6/7, HA-tagged constitutively active ALK1-7, kinase defective ALK3/4 as well as Ad-LacZ adenoviruses were used in these studies. The recombinant adenoviruses Ad-CAGA$_9$-luciferase [208] and the wild-type HA-tagged Ad-ALK5 were provided by Dr. Peter ten Dijke (The Netherlands Cancer Institute). The Ad-ALK4 adenovirus was produced using the Transpose-Ad system (Q-Biogene Illkirch, France) based on reference [209]. The ALK4-coding sequence was obtained from the plasmid pCIAlk4 (ActRIB), provided by Dr. A. Klibanski (Massachusetts General Hospital, Boston, MA).

The original cloning and generation of the recombinant Ad-Smad1/2/6/7 as well as the constitutively active and kinase defective Ad-ALK-adenoviruses has been reported earlier (Fujii et al., 1999). Briefly, a kit based on a previously described method (Miyake et al., 1996) for making recombinant human serotype 5 adenoviruses was purchased from Takara Biomedicals (Takara Shuzo Co., Shiga, Japan). The respective Smad and ALK cDNAs were cloned into the vector pAxCAwt, which contains a potent β-actin promoter and cytomegalovirus (CMV) enhancers. After preparation of cosmids in competent cells (*Escherichia coli*), co-transfection of the cosmids with adenoviral DNA into 293T cells was performed, after which recombinant adenoviruses were generated through homologous recombination. The recombinant adenovirus vector used lack the E1 and E3 genes needed for propagation of the virus. However, these genes are provided by 293T cells, hence the adenoviruses can propagate in them.

9. Adenovirus amplification and purification

For the generation of high titer adenovirus stocks, HEK-293T cells were seeded on gelatin-coated culture flasks (Greiner Bio-One). At 50-70% confluency (within 1-2 days),

the cells were infected with the recombinant adenovirus. Within 2-3 days the cells started to detach and show cytopathic effects, after which they were harvested and lysed using repeated freezing and thawing. Amplification steps were repeated three times and for the final round of amplification twelve 175 cm2 culture flasks were used. Purification of the adenoviruses from the final pool of lysed cells was achieved by ultracentrifugation for 90 min through CsCl gradients of densities 1.3 and 1.6. The virus band was retrieved and purified using PD-10 columns (Pharmacia) and the fraction containing the virus was determined using spectrophotometry. Virus aliquots were stored at -80°C in PBS containing 10% glycerol. Titrations of the recombinant adenoviruses were performed in 293T cells according to the TCID50 method described in the adenovirus manual supplied by Takara Biomedicals (http://bio.takara.co.jp/BIO_EN/default.asp). All adenoviral work was carried out in a P2 level classified laboratory.

10. Adenovirus infections

The use of recombinant adenoviruses in hGL cultures has been recently optimized [210]. We found in these previous studies that cytopathic effects are seen when purified recombinant adenoviruses are added to hGL cells at multiplicity of infection (MOI) values approaching 300. These effects are characterized by cellular detachment and death.

Before adenovirus infection, hGL cells were cultured for 2–3 days. The hGL cells were infected by incubating with the virus(es) at 37°C in serum-free DMEM supplemented with L-glutamine and antibiotics for 1 h, and DMEM containing 2% FCS was added on top to stop the infection. The cells were then incubated for 24 h before continuing the experiments. For details of individual experiments, please refer to the individual studies I-IV and references.

11. Transient transfections and luciferase assays

HEK-293T cells were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO2. The 293T cells were plated at low confluence on 24-well plates and grown overnight before transfections. Transfections were performed in 0.5 ml medium with 100 ng/well $(CAGA)_{12}$-luciferase reporter construct [205] or the BMP response element (BRE)-luciferase reporter construct [206] and 10 ng/well β-galactosidase reporter plasmid using the FuGene 6 transfection reagent Twenty-four hours later the cells were treated with TGF-β, activin A, BMP-2, or GDF-9 in 1% FCS/DMEM for 24 h. The cells were then lysed into 1X passive lysis buffer, and luciferase activity was measured with luciferase assay reagent (Promega Corp., Madison, WI) and normalized to β-galactosidase activity. Data are the mean ± SEM of triplicate determinations from representative experiments, relative to an adjusted value of 1.0 for the mean of the control wells without ALK4 or ALK5 adenoviral infection.

Rat granulosa cells (3 × 105 viable cells/well) were cultured in 24-well plates in McCoy's 5a medium supplemented with 10% FCS for 3 h. After media change, cells were incubated in the serum-free medium and transfected with 300 ng of DNA per well using Lipofectamine 2000 (Invitrogen). The pCMV-β-galactosidase plasmid was cotransfected to monitor transfection efficiency. After transfection, cells were treated with the appropriate ligand for 24 h in McCoy's 5a medium/1% FBS.

For the siRNA (small interfering RNA) experiments, 250 ng of DNA were transfected with or without increasing amounts of siRNAs together with Lipofectamine 2000 (Invitrogen). Twenty- four hours after transfection, cells were treated with the appropriate ligand for 24 h in McCoy's 5a medium/1% FBS. To harvest cells, lysis buffer (200 µl) (Promega Corp.) was added into each well and 30 µl of the supernatant was used for luciferase determination using a luminometer (Luminark microplate reader, Bio-Rad Laboratories, Inc.). The β-galactosidase activity was also determined to monitor transfection efficiency. The reporter activity is expressed as the ratio of relative light unit/β galactosidase activity. Data are the mean ± SEM of triplicates from representative experiments.

P19 cells were cultured in α-MEM supplemented with 10% fetal bovine serum (FBS), together with 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. Cells were seeded at 60% confluency in 24-well plates and transiently transfected in α-MEM for 4 h with 1.8 µg of DNA per well using FuGene-6. After transfection, cells were treated with the appropriate ligands for 24 h in α-MEM containing 5% FBS. COS7 cells were cultured in high glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. COS7 cells were seeded at 90% confluency in 24-well plates and transiently transfected in DMEM/ high glucose for 4 h with 1 µg of DNA per well using Lipofectamine 2000.

12. siRNA

The siRNAs were chemically synthesized by QIAGEN (Valencia, CA). The siRNA sequences targeting ALK5 corresponded to the nucleotides 1399–1421 of the rat ALK5 sequence (Gen- Bank accession no. L26110) 5'-AACAGATGGCAGAGCTGTGAGGC-3'. The control siRNA 5' AATTCTCCGAACGTGTCACGT- 3' was from QIAGEN. For annealing of siRNAs, 20 µM single-strand RNA were incubated in the annealing buffer [100 mM potassium acetate, 30 mM HEPES-KOH (pH 7.4), 2 mM magnesium acetate] for 1 min at 90°C followed by 1 h at 37°C.

13. RNA extraction and preparation of filters

Cytoplasmic RNA from freshly isolated hGL cells was extracted with the modified Nonidet P-40 lysis procedure [211, 212]. RNA samples were quantitated by absorbance measurement at 260 nm. For Northern blots, indicated amounts of RNA were size-fractionated in 1.5% agarose gels, after which they were transferred to Hybond-N nylon filters.

14. Preparation and labelling of cDNA probes and hybridizations

For the detection of inhibin α- and βB-subunit mRNAs by Northern Blot hybridization, double- and single-stranded cDNA probes were prepared as previously described [211, 212]. A human cyclophilin cDNA was used as a loading control for the experiment [211]. Northern blot hybridizations were performed for 16 h at 42°C, and the filters were washed three times for 20 minutes each time with 0.1-1 X standard saline citrate/1% sodium dodecyl sulfate at 50°C. The filters were exposed to a Fujifilm Ip-reader Bio-Imaging Analyzer Bas 1500.

15. Western blotting

Prior to Western blotting, the cells were washed once on ice with PBS, after which they were harvested in 1× Laemmli-buffer containing 10% β-mercaptoethanol or DTT as a reductant. Cells were sonicated on ice for 10 s with a Soniprep 150 MSE sonicator (Sanyo Corp., Japan) and boiled for 3 min. Proteins were separated on 10-15% SDS-PAGE gels and electroblotted onto Hybond-C membranes (Amersham). For the detection of hemagglutinin (HA)-tagged ALK receptors a monoclonal rat anti-HA antibody (Boehringer Mannheim, Indianapolis, IN, USA; clone 3F10) was utilized, and for the detection of FLAG-tagged Smads a monoclonal anti-FLAG antibody (Sigma; clone M2) was used. The commercial antibodies were used according to protocols provided by the manufacturers. For the detection of C-terminally phosphorylated Smad1 and Smad2, α-phospho-Smad1 or α-phospho-Smad2 antibodies were used. Phospho-Smad3 antibody was provided by Dr. E. Leof (Mayo Clinic, MN).

16. Enzyme-linked immunosorbent assay (ELISA)

Commercial ELISAs for the detection of immunoreactive dimeric inhibin A and B in the spent granulosa cell media were used according to the manufacturer's (Serotec Ltd., Oxford, UK) instructions. The assays use monoclonal antibodies directed toward the human inhibin βA- and βB-subunits for capture and a Fab alkaline phosphatase conjugated monoclonal antibody against the human inhibin α-subunit for detection. The crossreactivity between the two assays is minimal, approximately 0.5% for inhibin A in the inhibin B ELISA and < 0.1% for inhibin B in the inhibin A ELISA [213, 214]. A signal amplification kit (Life Technologies) was used in connection with the ELISAs. To achieve concentrations within the measuring ranges of the assays the spent culture medium was diluted with fresh medium.

RESULTS AND DISCUSSION

1. Granulosa cell model and adenoviruses

We chose to use human granulosa luteal (hGL) cells as our cell model for studying the interactions and biological functions of the TGF-β superfamily ligands and receptors, since we had access to these cells through the infertility clinics mentioned earlier in the material and methods section. Some granulosa cell lines have been developed from ovarian tumours (e.g. the COV434 cell line, [215], and the KGN cell line, which expresses FSH receptors and is responsive to FSH [216]), but these were not available for our use when this thesis work was started.

The hGL cells retrieved from hormone stimulated patients come from pre-ovulatory follicles and are as such refractory to further gonadotropin stimulus. However, they regain the responsiveness to hCG and FSH after about 2-7 days of culture and produce progesterone as well as estradiol under gonadotropin stimulation [217]. The human granulosa cells have been shown to express the TGF-β superfamily type I receptors ALK2 [218], ALK3 [219], ALK4 [218] and ALK5 [220]. Also the type II receptors BMPRII [219], TβRII [220], ActRIIA [218] and ActRIIB [218] as well as Smad1, -2, -3, -4, -5 and -6 [219] are expressed in these cells. ALK1 is a TGF-β1 receptor specifically expressed in endothelial cells [44, 221], and its expression has not been reported in hGL cells. ALK7 expression has been detected in the ovary, but not reported specifically in granulosa cells [222]. Thus, most, if not all, of the TGF-β superfamily signalling components are present in hGL cells, which makes these cells a good model for studying the mechanisms of TGF-β superfamily ligand signal transduction in an ovarian setting.

1.1. Adenoviral gene transfer to cultured human granulosa-luteal (hGL) cells

The human granulosa luteal cells are terminally differentiated and cannot divide in culture conditions, although with the addition of special serum these cells may be stimulated to proliferate [223]. However, in our hands, the proliferating cells derived from the primary hGL cells lost some of their characteristics, such as responsiveness to GDF-9 (our unpublished results). Human granulosa luteal cells are also very difficult to transfect by conventional methods. Previously, adenovirus-mediated gene transfer has been successfully used in cultured rat granulosa cells [224, 225]. We therefore infected hGL cells with a recombinant adenovirus that expresses the green fluorescent protein (GFP) under cytomegalovirus (CMV) promoter to find out whether the hGL cells were susceptible to adenoviral infection. Twelve hours after the infection, the cells were monitored with a fluorescence microscope (I: Figure 1A-C). Already with multiplicity of infection (m.o.i.) values of 1 to 3 many cells expressed GFP. At m.o.i. values above 10, the majority of cells were GFP positive. M.o.i. values exceeding 300 caused a cytopathic effect

due to the adenovirus infection and the cells started to detach and die. Therefore, m.o.i. values between 1 and 100 were chosen for our following experiments. Thus, adenoviral transduction provides an efficient method to introduce genes of interest also into the hGL cells.

1.2. Recombinant adenoviruses

Since their discovery in 1953 [226], adenoviruses have become a versatile model for elucidating elementary aspects of gene expression in mammalian cells, leading for example to the discovery of mRNA splicing. Today, adenoviruses are commonly used as gene transfer vehicles due to their ability to infect a wide range of non-dividing cells. Adenoviruses are nonenveloped icosahedral double stranded DNA viruses with a linear genome of ca. 36-kbp. They are known to cause a variety of human diseases including respiratory tract infection, conjunctivitis and gastroenteritis. More than 100 different adenovirus species have been identified from mammals, birds and reptiles. These viruses are classified into six subgroups (from A to F), which are further divided into serotypes based on their immunological properties. Serotype 2 and 5 of subgroup C are the most extensively studied of the human adenoviruses, and they are found worldwide. They bind to a primary receptor on the cell surface, the coxsackie-virus B adenovirus receptor (CAR) [227] which in turn activates integrin coreceptors leading to clathrin-mediated endocytosis of the adenovirus particle [228]. When the adenovirus genome has reached the nucleus, a group of 6 early transcription units (E1A, E1B, E2A, E2B, E3 and E4) are first transcribed. These early genes encode proteins involved in DNA replication, host immune system suppression (E3) and inhibition of apoptosis of the host cell. The E1A- and E1B-encoded products regulate cellular genes and transactivate the other early transcription units setting in motion the rest of the viral gene expression programme [229].

In the recombinant adenoviruses used in these studies, transcription units E1A, E1B as well as E3 have been deleted creating a genetic capacity of about 8 kb that can be used for DNA insertion. The resulting recombinant adenoviruses are helper-independent but can proliferate only in transcomplemental cell lines such as 293 or 911 cell lines that express the deleted viral genes (reviewed in [230]).

2. Activin and BMP signalling pathways are involved in the induction of inhibin B protein secretion in hGL cells (I)

Activins and inhibins were first discovered as gonadal proteins that regulate pituitary FSH secretion. Three types of activin can be produced in the ovary by the granulosa cells, each consisting of a dimer of two related subunits, inhibin βA and inhibin βB. Homodimeric activin A therefore contains two inhibin βA subunits, whereas and activin B consists of two inhibin βB subunits. The heterodimeric activin AB is formed from one βA and one βB subunit. Activin produced by the secretory gonadotrophs in the anterior pituitary stimulates FSH production in a paracrine manner, and within in the ovary

activin promotes granulosa cell proliferation and potentiates FSH actions by increasing FSH receptor expression.

Two types of inhibins are also expressed by granulosa cells. Inhibins consist of one inhibin β-subunit and one inhibin β subunit thus forming either inhibin A (α-βA) or inhibin B (α-βB). Inhibins are also produced by the granulosa cells, and they act as endocrine hormones that are released into the circulation to suppress pituitary FSH production. Locally, inhibins also act as potent regulators of activin signalling. Inhibins compete activin signalling by blocking activin binding to type II activin receptors. β-glycan, an inhibin co-receptor, facilitates inhibin binding to the activin type II receptor.

Inhibin and activin synthesis in the ovary depends on the regulation by the pituitary gonadotropins as well as by local paracrine factors. The hGL cells from preovulatory follicles express inhibin α and inhibin βA subunit mRNA, whereas inhibin βB subunit can not be readily detected [212]. Gonadotropins have been shown to stimulate the expression of inhibin α subunit mRNA within 24-48 hours and inhibin βA subunit mRNA within 2 hours as well as the secretion of dimeric inhibin A and B proteins by hGL cells [231, 232]. The expression of the inhibin α subunit decreases during prolonged culture of these cells whereas the levels of βA subunit remain unchanged [212]. In addition to the gonadotropins, TGF-β1 and -2 as well as activin A and BMP-2 can induce the inhibin βB subunit mRNA expression in cultured hGL cells [219, 232, 233].

The gonadotropins mediate the induction of inhibin and activin subunits through a cAMP-PKA pathway in the ovarian granulosa cells [234] (see Figure 6 in the Review of the literature section for a schematic drawing of the signalling pathway), whereas the local paracrine factors appear to mediate their effect on inhibin production through the Smad pathways (Studies I and II).

To study the role of the TGF-β superfamily receptors and intracellular signalling molecules in the induction of inhibin βB subunit expression as well as the dimeric inhibin B protein production by these ligands, we transduced hGL cells with recombinant adenoviruses expressing the individual constitutively active type I receptors ALK1 through ALK7 as well as Smad1 and -2. ALK1, -2, -3 and ALK6 are known to activate Smad1, -5 and -8 of the BMP-pathway, whereas ALK4, -5 and ALK7 activate the TGF-β/activin pathway Smads, Smad2 and -3. The expression of all the ALK-receptors was detected using α-HA immunoblotting whereas the Smad proteins were detected using α-FLAG immunoblotting.

2.1. The expression of constitutively active ALKs activates Smad1 and -2 pathways

Recombinant adenoviruses were used to express constitutively active (ca)-ALKs to enable us to follow the activation of Smad signalling proteins in hGL cells. The ca-ALKs have distinct point mutations in their kinase domains that enable them to phosphorylate their downstream signalling molecules, the Smads, independently of ligand binding [235]. Cultured hGL cells were infected with Ad-ca-ALK1-7, and as expected, adenovirally expressed constitutively active ALK1, -2, -3 and -6 could specifically activate both the

endogenous and exogenously expressed Smad1 signalling pathway which was detected with a rabbit polyclonal antibody raised against the C-terminal phosphopeptide of Smad1 (αPS1) (I: Fig. 2A and 2B, respectively).

Ca-ALK4,-5 and -7 activated both the endogenous and exogenous Smad2 protein in hGL cells. The activation was detected by utilizing a rabbit polyclonal antibody raised against the C-terminal phosphopeptide of Smad2 (αPS2), (I: Fig. 2A and 2C, respectively). The αPS1 and αPS2 antibodies are specific to the phosphorylated forms of the Smad proteins and do not recognize the unphosphorylated proteins. However, the αPS1 antibody is known to cross-react also with the phosphorylated forms of Smad5 and -8 as well as Smad3 (Peter ten Dijke, unpublished data). Because of this, additional bands that can be seen in Figures 2A and 3A (I) might represent these other Smads.

In addition, BMP-2 could activate Smad1, with the amount of phosphorylated Smad1 peaking at 45 minutes and declining thereafter. Correspondingly, activin A was able to activate Smad2 protein causing a peak in phosphorylation of Smad2 after 30 minutes which started to decline after 60 minutes (I: Fig. 3A and 3B, respectively).

2.2. The activation of Smad1 and Smad2 pathways promotes inhibin B protein secretion in hGL cells

Overexpression of each of the constitutively active ALKs promoted inhibin B protein secretion through the activation of Smad1 and Smad2 signalling proteins as measured by specific inhibin B ELISA. Inhibin B protein concentration was increased in the spent hGL media within 48 h after infection with ca-ALK1-7 when compared to controls (I: Fig. 4A) while control viruses had no effect. Ca-ALK3 and ca-ALK4 viruses significantly stimulated the production of dimeric inhibin B within 48 hours depending on the amount of virus (I: Fig. 4B(1-2), and 4C(1-2)). Infection with increasing amounts of the Smad1 and Smad2 viruses caused a corresponding increase in the phospho-Smad1 and phospho-Smad2 levels, as detected by αPS1 and αPS2 antibodies (I: Fig. 5C-D, respectively for Smad 1 and Smad2) and caused a dose-dependent increase in the secretion of inhibin B (I: Fig. 5A and 5B, respectively).

This is in line with earlier results showing that stimulation with either TGF-β/activin or the BMPs results in the induction of inhibin B secretion in the hGL cells and indicates that the Smads participate in the regulation of inhibin production in these cells [219, 233, 236]. Interestingly, no ligand-induced activation of the Smads was required to induce the phosphorylation, but the Smads were spontaneously activated when overexpressed. A similar phenomenon has been described before by others for Smad3 [237-239]. It has been suggested that the overexpression of Smad3 could overcome the ability of SARA (Smad anchor for receptor activation) to bind monomeric unphosphorylated Smad3 and prevent the formation of activated trimers in the absence of ligand stimulation [240]. A similar mechanism might also regulate the ligand-independent activation of Smad1 and Smad2.

2.3. Kinase defective ALK3 and ALK4 and the inhibitory Smad7 inhibit activin and BMP stimulated inhibin B production in hGL cells

Human granulosa luteal cells were infected also with adenoviruses expressing kinase defective (kd)-ALK3 and -ALK4 before stimulation with 25 ng/ml BMP-2 or activin A. The concentration of dimeric inhibin B protein was measured 3 days later in the spent media. The kinase defective type I receptors act in a dominant negative way and block the signalling pathway by inhibiting the activation of the downstream Smad proteins. Kd-ALK3 blocked the stimulatory effect of BMP-2, whereas kd-ALK4 blocked the stimulatory effect of activin A on the production of inhibin B dimers (I: Fig. 6A and 6B, respectively).

In addition, the BMP-2 and activin A stimulated inhibin B secretion was significantly decreased with the administration of increasing titres of the adenovirus expressing the inhibitory Smad7 (I: Fig 7). This result is in line with the agreement that Smad7 acts as a common inhibitor of both the TGF-β and the BMP ligands [241, 242], (whereas Smad6 is a specific inhibitor of the BMPs [243, 244]).

3. GDF-9 induces the expression of inhibin βB subunit mRNAs and secretion of dimeric inhibin B protein (II)

Previously, it had been reported that oocyte secreted factors stimulate inhibin A and B secretion in rat granulosa cells [245], and that TGF-β, activin and some BMPs induce inhibin production in hGL cells [219, 233, 236]. We therefore determined whether also GDF-9 had any effect on the inhibin production in hGL cells. We found that GDF-9 increased the inhibin βB-subunit mRNA steady state levels after 8 h of stimulation (II: Fig 3), whereas the inhibin α-subunit or inhibin βA-subunit transcript levels were not distinctly affected. However, in cultured rat granulosa cells from small antral follicles, GDF-9 has been shown to induce the transcription of the inhibin α as well as the inhibin βB subunits [246]. This discrepancy in the stimulation of inhibin α-subunit expression might arise from species specific differences in inhibin/activin production pattern or the different stage of follicular development of the granulosa cells.

The concentration of secreted dimeric inhibin B protein was measured from the spent media of untreated and GDF-9-stimulated cells using a specific inhibin B ELISA. GDF-9 stimulated the production of inhibin B within 24 h, with the maximal effects seen after 72 h (II: Fig. 3B). GDF-9 induced the inhibin B production in a concentration-dependent manner, with maximal stimulations seen at 300 ng/ml GDF-9 (II: Fig. 3C). GDF-9 has previously been shown to induce the secretion of both inhibin A and B in the presence or absence of FSH in cultured rat granulosa cells from small antral follicles [246]. Similarly, denuded bovine oocytes have been shown to produce factors that stimulate inhibin A and B protein production, as do TGF-β and activin in rat granulosa cells [245].

Since Ad-Smad7 suppressed inhibin B production stimulated by BMP-2 and activin A [210], we determined whether overexpression of this inhibitory Smad would also affect GDF-9-stimulated inhibin B production. Increasing amounts of Ad-Smad7 (m.o.i. values of 0.3–30) suppressed GDF-9- induced dimeric inhibin B production in hGL cells dose-

dependently (II: Fig. 4, A and C), whereas Ad-Smad7 alone did not affect basal inhibin B levels (II: Fig. 4, B and D). These results suggest that the Smad pathway could be involved in the GDF-9-stimulated inhibin B production.

Small antral follicles are thought to be the principal expression site of the inhibin βB subunit during folliculogenesis [247-249], which is supported by the increase of circulating inhibin B protein levels in the serum at the beginning of the follicular phase during the human menstrual cycle [213]. Therefore, the luteinized human granulosa cells do not seem to be the physiological producers of inhibin B, and caution should be kept when considering the physiological relevance of these results. However, inhibin B production is a convenient parameter to measure when assessing the bioactivity of GDF-9 or other superfamily ligands or their signalling pathway in our granulosa cell model.

4. GDF-9 activates both Smad2 and Smad3 but not Smad1 pathway in ovarian granulosa cells, HEK 293T as well as the P19 cell line (II-IV)

4.1. Recombinant GDF-9 induces endogenous αPS2 immunoreactive proteins in hGL cells

As a member of the TGF-β superfamily, GDF-9 as well, could be predicted to signal through the canonical TGF-β superfamily receptor-Smad pathways, and the first evidence of this was the report stating that GDF-9 was able to bind the type II receptor BMPRII [42]. Because of this it was anticipated that GDF-9 would signal through the BMP-pathway Smads.

We established a stable cell line producing the untagged recombinant mouse GDF-9 and raised a high affinity monoclonal antibody against the C-terminal domain of human GDF-9 to verify the presence of GDF-9 in the 293T cell-production medium. The antibody against human GDF-9 was named mAb #37. It detected both the rat and mouse recombinant GDF-9 in Western blots analysis of the culture medium of GDF-9-expressing 293T cells (II: Figure 1A). The 293T cell-produced mouse GDF-9 seems to be fully processed, whereas the rat GDF-9 consists of the processed mature region as well as the unprocessed protein precursor. Both GDF-9 proteins are, however, equally potent stimulants when similar amounts of processed mature region proteins are present in the 293T-conditioned medium.

We first studied which of the Smad pathways, the TGF-β/activin or the BMP pathway, was activated by GDF-9 in hGL cells. We followed the activation of the two Smad pathways under GDF-9 stimulation by Western blot analyses using the αPS1 and αPS2 antibodies. With GDF-9 stimulation, αPS2-reactive bands first appeared within 30 min of stimulation and peaked within 75 min (II: Fig. 1B, top panel) whereas no immunoreactivity was detected in controls. A similar time course for Smad activation has been observed by others in different types of cultured cells [250].

Induction of the αPS2-immunoreactive proteins by GDF-9 was dose-dependent, and the strongest effect was seen with 150 ng/ml of GDF-9 (II: Fig. 1B, lower panel). The

upper panel of Fig. 1C (II) shows that, like TGF-β, GDF-9 induced the appearance of αPS2-immunoreactive protein. αPS1-immunoreactive proteins were induced by BMP-2 but not by TGF-β or GDF-9 (II: lower panel of Fig. 1C).

The αPS2- and αPS1-immunoreactive bands correspond to the predicted molecular masses (~53 kDa) of the phosphorylated Smad2 and Smad1, respectively. It can be therefore concluded that GDF-9 uses the same signalling pathway as TGF-β and activin, being able to induce the activation of Smad2 pathway instead of the BMP-Smad pathway in the human ovarian granulosa cells, despite the fact that it binds the type II BMP receptor [42]. The ability of GDF-9 to induce Smad2 activation was also tested in another cell model, the P19 cell line. GDF-9 treatment stimulated the phosphorylation of Smad2 protein but not Smad1, in P19 cells similar to activin (III: Fig 2B). Both these results are in line with findings that GDF-9 could activate the Smad2 pathway in cultured rat granulosa cells from small antral follicles [246].

4.2. GDF-9-induced phosphorylation Smad2 in hGL cells is inhibited by Ad-Smad7

To verify that GDF-9 is actually able to induce phosphorylation of the Smad2 protein, we introduced an exogenous Smad2 gene into hGL cells through adenovirus infection. Recombinant GDF-9 could stimulate the phosphorylation of both the endogenous and the adenovirally produced Smad2 proteins (II: Fig. 2A). At m.o.i. values of 5 or lower, αPS2-reactive bands were rarely seen in unstimulated cells, whereas with Smad2 viruses given at m.o.i. values of 10–100, spontaneous activation of Smad2 phosphorylation was clearly detected. This phenomenon has also been reported by others as R-Smad proteins that are overexpressed excessively from expression plasmids have been shown to be spontaneously activated in other types of mammalian cells as well [237-239]. Therefore, Ad-Smad2 m.o.i. values of 3–5 were chosen for most experiments to avoid excessive Smad2 expression.

We next introduced increasing amounts of Ad-Smad6 or Ad-Smad7 (m.o.i. ranging from 0.3–30) into hGL cells together with a constant amount of Ad-Smad2 (m.o.i. 5) to see whether the inhibitory Smads could block the GDF-9-induced Smad2 phosphorylation. The GDF-9-induced phosphorylation of both the endogenous and the adenovirally expressed Smad2 proteins was gradually reduced close to the control level in cells infected with Ad-Smad7 (II: Fig. 2B), whereas Ad-Smad6 was very ineffective in suppressing Smad2 phosphorylation (II: Fig. 2C). This is in line with earlier findings that Smad7 is a universal inhibitor of the TGF-β superfamily ligands and that Smad6 functions specifically as a BMP-inhibitor [241-244].

These results suggest that functionally, GDF-9 should be classified as a TGF-β/activin-like growth factor rather than a BMP-like factor, although phylogenetically GDF-9, as well as its close homologue GDF-9B, are closer to the BMP-subgroup than the TGF-β/activin-subgroup [251]. The phylogenetic relationship to the BMPs would also be supported by the fact that the type II receptor for GDF-9, as well as for GDF-9B, is the type II receptor for the BMPs, BMPRII [25, 42].

4.3. Activation of Smad3-specific reporter and phosphorylation of Smad3 in HEK-293T and P19 cells by GDF-9

Since Smad2 was activated by GDF-9 stimulation in hGL cells, we investigated whether GDF-9 could also activate the Smad3 protein. We used plasmid reporter constructs that are specific for the TGF-β/activin Smad3 pathway (CAGA-luciferase) or the BMP Smad1/5/8 pathway (BRE-luciferase) in HEK 293T cells. The CAGA-luciferase reporter construct consists of twelve tandem repeats of the Smad binding element located in the plasminogen activator inhibitor (PAI) promoter, and is specifically activated by phosphorylated form of Smad3, downstream of ALK4, -5 and -7 receptors [205]. It is not activated by the phosphorylated Smad2.

The BRE-luciferase reporter construct contains a BMP-specific response-element from the Id1 (inhibitor of differentiation 1) promoter and is specifically activated by Smad1, -5 and -8 [206]. We found that the HEK-293T cell line was very responsive to GDF-9, and the responsiveness was measured by the induction of the CAGA-luciferase reporter activity (IV: Fig. 1A). In the same cell line, the BMP-responsive reporter construct, BRE-luciferase, was only activated by BMP-2 and not by GDF-9 (IV: Fig. 1B). GDF-9 activated the CAGA promoter in 293T cells dose-dependently (IV: Fig. 1C).

These results were repeated in another cell line, the mouse embryonic carcinoma-derived P19 cells. As shown in Fig. 1A (III), treatment of P19 cells with GDF-9 induced the activation of the CAGA luciferase reporter like TGF-β and activin. Although BMP-2 was ineffective on the CAGA promoter (III: Fig. 1A), treatment with BMP-2, stimulated the reporters driven by the BRE or the GCCG-promoter whereas GDF-9, TGF-β, or activin had no effect (III: Fig. 1, B and C, respectively). Furthermore, GDF-9 stimulation of the CAGA promoter was dose-dependent (III: Fig. 1D). These findings suggest that also P19 cells express the receptors and downstream effectors that are required for GDF-9 signalling.

Because GDF-9 was able to activate the Smad3-dependent CAGA promoter, we also examined whether it could cause the phosphorylation of Smad3 protein in GDF-9-treated 293T and P19 cells. As expected, GDF-9 increased the level of phosphorylated recombinant Smad3 (IV: Fig. 1D) in 293T cells. GDF-9 also increased the level of phosphorylated Smad3 in the P19 cells as shown in Fig. 2 A (III).

4.4. Activation of a Smad3-specific reporter in hGL and rat granulosa cells by GDF-9, TGF- and activin and phosphorylation of Smad3 in rat granulosa cells

Because we had observed the activation of Smad3 by GDF-9 in human 293T cells and P19 cells (IV: Fig. 1D and III: Fig. 2), we wanted to determine whether GDF-9 activated Smad3 in primary cultures of hGL cells (II: Fig. 1B and C). We introduced an adenovirus expressing a $(CAGA)_9$-luciferase into hGL cells, and stimulated the cells with different ligands. GDF-9, TGF-β, and activin all activated the $(CAGA)_9$-luciferase reporter in hGL

cells dose-dependently (IV: Fig. 2) whereas BMP-2 did not (IV: Fig. 2C) demonstrating the specificity of the assay for Smad3-activating ligands. GDF-9 treatment also increased the CAGA promoter activity dose-dependently in granulosa cells obtained from early antral follicles of immature rats transfected with the CAGA-promoter, as did activin and TGF-β (III: Fig. 7A and B).

Our earlier data had demonstrated the ability of GDF-9 to stimulate Smad2 phosphorylation in rat granulosa cells [246] and because studies using P19 cells demonstrated the ability of GDF-9 to stimulate Smad3 phosphorylation, we tested whether treatment with GDF-9 could induce the phosphorylation of Smad3 proteins also in rat granulosa cells. As shown in Fig. 7C (III), treatment with GDF-9, like activin, increased the level of phosphorylated Smad3 protein. These data suggested that the GDF-9 stimulation of the CAGA promoter is correlated with its stimulation of Smad3 phosphorylation in granulosa cells.

4.5. GDF-9 stimulation of the CAGA promoter is blocked by the inhibitory Smad7 but not by Smad6 in both rat granulosa cells and the P19 cells

Because the transcriptional activities of all R-Smad proteins (Smad1, -2, -3, -5, and -8) are blocked by the inhibitory Smad7, whereas those of Smad1, -5, and -8 are blocked by the inhibitory Smad6, we tested the inhibitory activities of the two inhibitory Smad proteins in P19 cells and rat granulosa cells treated with GDF-9. Figure 3A (III) shows that a cotransfection with Smad7, but not Smad6, led to a dose-dependent suppression of the GDF-9 stimulation of the CAGA promoter together with a minor inhibition of the basal promoter activity. Consistent with the role of Smad1, 5, and 8 in BMP-2 signalling, treatment with either Smad6 or Smad7 blocked the BMP2 stimulation of the BRE promoter in the same cells (III: Fig. 3B).

These results suggest that GDF-9 signalling does not involve the BMP-responsive pathway mediated by Smad1, 5, and 8 and likely involves Smad2 and Smad3 activated by ALK4, 5, or 7. We further tested the effect of inhibitory Smad proteins on GDF-9 signalling in the rat granulosa cells. As shown in Fig. 8 (III), transfection of these cells with the inhibitory Smad7 suppressed GDF-9 stimulation of the CAGA promoter, consistent with our findings derived from P19 cells. In contrast, only minimal suppression of GDF-9 effects was observed when cells were transfected with the highest dose (150 ng) of Smad6.

Both Smad2 and Smad3 are expressed in the ovary at the stages of follicular development when GDF-9 is expressed [252]. Both Smads are present in rat preantral follicles but disappear from large antral follicles. Smad2 expression returns in luteal cells, whereas Smad3 remains absent. Smad2 has been shown to be more responsive to activin and Smad3 to TGF-β signalling in rat granulosa cells [252]. Stage-specific expression and differing ligand sensitivity of signalling molecules may work together to allow different effects of TGF-β family ligands using the same signalling pathways over the course of follicular development.

5. GDF-9 signalling is mediated by the type I receptor ALK5 (III and IV)

The type II receptor for GDF-9 was discovered in 2002 by Vitt et al. The BMP type II receptor (BMPRII) soluble ectodomain was shown to be most effective in blocking the GDF-9-induced granulosa cell proliferation and suppression of FSH-induced progesterone production [42]. In addition, GDF-9 showed a direct interaction with BMPRII ectodomain in a co-precipitation study, whereas ActRIIA ectodomain showed only minimal interaction and could only partially block granulosa cell proliferation [42]. The BMP type I receptors ALK3 and -6 also partially inhibited the GDF-9-induced granulosa cell proliferation, whereas ALK2 had no effect. In binding studies, none of these ALKs showed interaction with GDF-9. These results with the BMP ALKs are curious, since sequence alignment of GDF-9 had revealed that it is most closely related to the BMPs, and therefore might have been thought to signal via the BMP pathway receptors and Smads.

A close homologue of GDF-9, GDF-9B, has also been shown to bind BMPRII but has high affinity to ALK-6 [25]. GDF-9B induces the activation of Smad1/5/8 pathway but curiously is also able to activete the Smad2/3 pathway in COV434 cells, although weakly [25]. Thus, knowing that GDF-9 induces a TGF-β/activin-like response in stimulating the activation of Smad2/3 pathway, the group of type I receptors capable of mediating the GDF-9 signal would be ALK4, -5 or -7. Therefore, the following studies were conducted to elucidate the type I receptor for GDF-9.

5.1. Adenoviral overexpression of wild-type ALK5 and ALK4 amplifies the CAGA-luciferase response of hGL cells to GDF-9/TGF-β and activin, respectively

We studied the effect of adenoviral-mediated overexpression of either ALK5 or ALK4 in hGL cells on the GDF-9, TGF-β, or activin response, and measured their effects in the Smad3-responsive CAGA-luciferase reporter assay. Overexpression of ALK5 enhanced the response of hGL cells to GDF-9 and TGF-β stimulation dose-dependently but not to activin (IV: Fig. 3A). In contrast, the overexpression of ALK4 did not affect the GDF-9 response of the transduced hGL cells, but caused a strong dose-dependent response to activin in these cells (IV: Fig. 3B).

5.2. Effect of overexpression of type I receptors Alk5 and Alk4 in hGL cells on GDF-9-induced inhibin B production

We have shown that GDF-9 stimulates the production of inhibin B by hGL cells and rat granulosa cells ([246] and study II). Therefore we examined the effect of overexpression of either ALK5 or ALK4 in hGL cells on the GDF-9 response, and measured the level of inhibin B in the culture medium from the adenovirally transduced cells. Overexpression of ALK5 caused a strong potentiation in the response of hGL cells to GDF-9 (IV: Fig.

4A), whereas the TGF-β-stimulated inhibin B response was less affected by the ALK5 expression level. The effect of overexpression of ALK4 on the GDF-9 response was minimal (IV: Fig. 4B). Overexpression of ALK4 alone was enough to cause an increase in the level of inhibin B produced by hGL cells, and the effect of GDF-9 treatment on these cultures was only additive in terms of inhibin B production.

5.3. Overexpression of ALK5 and Smad3 confers GDF-9 responsiveness in the nonresponsive COS7 cells

In preliminary tests, it was found that GDF-9 treatment could not activate the CAGA and BRE promoters in COS7 cells (III: Fig. 4A). However, these cells were responsive to TGF-β and activin based on the stimulation of the CAGA promoter (III: Fig. 4A), but also the BRE promoter-reporter was activated by BMP2 and BMP7 (III: Fig. 4B), suggesting that the COS7 cells express BMPRII to mediate BMP actions as well as ALK4, ALK5, and Smad3 to mediate the actions of TGF-β and activin. We hypothesized that the endogenous levels of one or more of the type I receptors, the type II BMPRII receptor, and/or Smad2 or Smad3 were too low in COS7 cells to allow efficient GDF-9 signalling. We therefore performed overexpression tests in COS7 cells in an attempt to gain GDF-9 responsiveness.

Transfection of these cells with increasing amounts of the ALK5 expression plasmid led to a dose-dependent stimulation of the CAGA promoter activity by GDF-9 (III: Fig. 5A). In these cells, transfection with the ALK5 plasmid also increased TGF-β, whereas activin signalling was not affected. However, transfection of increasing amounts of the ALK4 plasmid alone led to a dose-dependent increase in luciferase activity (III: Fig. 5B, left panel). In these ALK4-transfected cells, treatment with GDF-9 and TGF-) was ineffective in further stimulating the CAGA promoter, in contrast to activin treatment. In addition, overexpression of ALK7 did not confer hormonal responsiveness to GDF-9, activin, or TGF-β (III: Fig. 5C). In addition, overexpression of ALK1, 2, 3, or 6 did not confer GDF-9 activation of the CAGA promoter.

Because the CAGA promoter is responsive to Smad3, but not Smad2, activation (Table 1, III), we further tested whether transfection with Smad3, alone or together with ALK5, could render the COS7 cells responsive to GDF-9. As shown in Fig. 6 (III), cells overexpressing Smad3 did not show increased GDF-9 responsiveness. However, cotransfection of the Smad3 plasmid with a submaximal level (10 ng) of the ALK5 plasmid further augmented the GDF-9 stimulation of the CAGA promoter. These data suggested that Smad3 and ALK5 could act together to confer GDF-9 responsiveness to COS7 cells.

5.4. Inhibitory effects of ALK5 siRNA on GDF-9 signalling in granulosa cells

We further tested whether granulosa cells, like COS7 cells, require ALK5 for GDF-9 signalling. Control and ALK5 siRNAs were transfected into cultured granulosa cells before testing the ability of GDF-9 to activate the CAGA promoter. Small interfering RNAs act post-transcriptionally and degrade the targetted mRNAs in a sequence-specific manner causing the silencing of the intended gene [253]. As shown in Fig. 9A (III), transfection with increasing amounts of ALK5 siRNA led to dose-dependent decreases in CAGA promoter activity stimulated by GDF-9, whereas control siRNA had no effect. Treatment with ALK5 siRNA also suppressed CAGA promoter activity stimulated by TGF-β (III: Fig. 9B), while no inhibition of CAGA promoter activity by activin was observed (III: Fig. 9C). These findings demonstrated the important role of ALK5 in GDF-9 actions in granulosa cells.

6. GDF-9 signalling pathway

The receptor complex utilized by the oocyte-derived growth differentiation factor GDF-9 thus consists of the type II BMP receptor BMPRII and the activin-receptor like kinase ALK5. Upon GDF-9 binding and the formation of the active receptor-ligand complex at the plasma membrane, the intracellular effectors the Smads 2 and 3 are activated. These

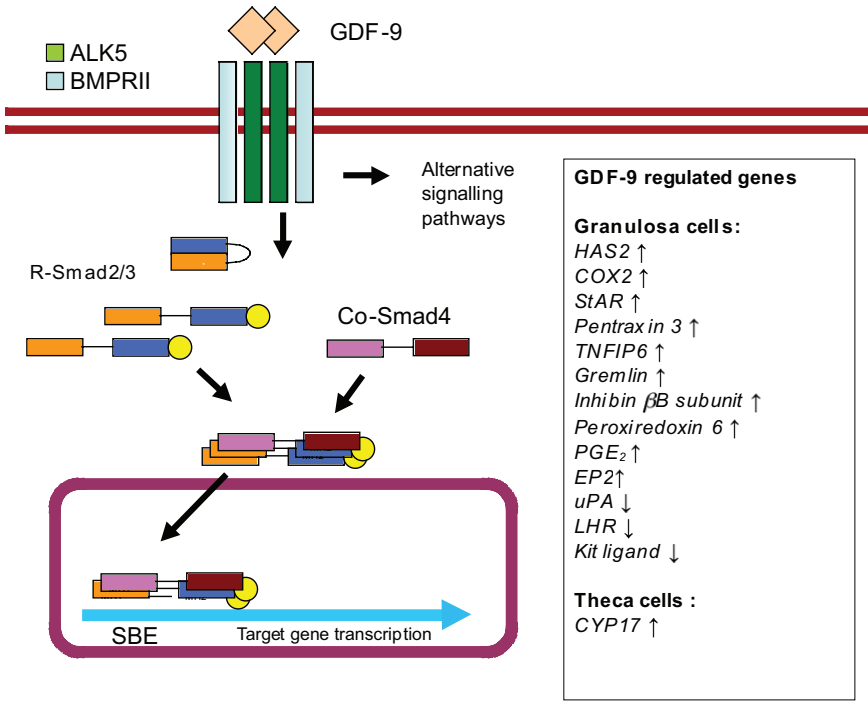

Figure 7. A schematic drawing of the Smad signalling pathway utilized by GDF-9.

proteins translocate into the nucleus in complex with the common-partner Smad4 to modulate target gene transcription (Figure 7).

The receptor combination utilized by GDF-9 is uncommon in that this case is the first time that BMPRII has been reported to associate with ALK5. This kind of cross-talk between the established TGF-β/activin and BMP pathway receptors is not unique, however, since TβRII can interact also with ALK1 in addition to ALK5 [254]. ALK5 had previously been shown to bind only TGF-β [51] and myostatin (GDF-8) [255].

Based on these studies, we can show that also GDF-9 can stimulate the activation of both the Smad2 and Smad3 proteins through ALK5. It is not known yet however, to what extent each of these Smads contributes to the modulation of target genes. In the case of TGF-β, it is known that the Smad2 and -3 have different downstram effects [256]. In addition, Smad2 knockout mice die early during embryogenesis [257, 258] while mice lacking functional Smad3 are viable. The different Smad3 knockout mice can display either impaired folliculogenesis due to diminished pool of follicles reaching the antral stage [259] or only smaller litter sizes compared to wild type mice depending on the location of disruption in the gene (deletions in exon 8 and exon 2, respectively) [260]. The delayed follicular maturation in Smad3 deficient mice with deletion in exon 8 could be caused by impaired GDF-9 signalling.

Our results concerning the signalling components and pathways have been obtained in *in vitro* culture conditions both in primary somatic ovarian cells and various stable cell lines. These cell models can not, however, mimic the physiological conditions within the ovary, and therefore these models can only give a rather static view of the signalling phenomenon. For example, the proper spatial and temporal dimensions of the signalling compoments and ligands are lacking in these models. Therefore, what we see is only an over-simplified picture. Gilchrist and his research group have utilized a different approach for studying the oocyte-derived growth factors and their signalling mechanisms. His group has used intact denuded oocytes (DO) from cumulus oocyte complexes (COC) and oocytectomized (OOX) cumulus cell complexes and under these conditions the physiological environment of the follicle may be better imitated.

In a recent article by Gilchrist et al. it was shown that the oocyte secreted factor(s) (OSF), most likely including both GDF-9 and GDF-9B, principally use the Smad2/3 pathway for stimulating granulosa cell growth [261]. Surprisingly, the BMP Smad pathway was not found to be activated despite the fact that the oocyte expressed growth differentiation factor GDF-9B is a granulosa cell mitogen [176] and is known to signal through the Smad1/5/8-pathway [261]. Also, the OSF seem to signal predominantly through the BMPRII receptor, since the soluble ectodomain of the BMPRII could block the oocyte-induced granulosa cell thymidine incorporation completely [261]. However, the oocytes used in Gilchrist's study [261] were derived from preovulatory antral follicles of immature mice primed with equine chorionic gonadotropin which may not express functional GDF-9B as is suggested by Yoshino et al. in their recent article. Yoshino et al. found that the functional mature form of GDF-9B is markedly expressed only just before ovulation in mouse oocytes, which could explain the absence of the BMP-Smad activation [181].

Yoshino also showed that recombinant GDF-9B is able to induce cumulus expansion in mouse cumulus-oocyte complexes [181] in the absence of FSH. GDF-9B stimulated the expression of EGF-like growth factors betacellulin, epiregulin and amphiregulin in cumulus cells as well as a series of molecules downstream of EGF-like growth factor signaling, including cyclooxygenase 2 (COX2), hyaluronan synthase 2 (HAS2), tumor necrosis factor-stimulated gene 6 (TNFIP6), and pentraxin 3 (PTX3), all of which are necessary for normal cumulus expansion.

Cumulus expansion has now been shown to be induced by both of the oocyte-expressed growth factors, GDF-9 [138, 170] and GDF-9B [181], in addition to FSH or epidermal growth factor (EGF) [262, 263]. The combined stimulation by these aforementioned factors results in the activation of MAPK3/1 (ERK1/2) and MAPK14 (p38) proteins [264, 265] to enable the up-regulation of downstream targets HAS2, COX2 (Ptgs2), TNFIP6 and PTX3 required for expansion [266]. Thus, the oocyte-enabled activation of the MAPK pathway in cumulus cells is required for their expansion [264], and inhibition of the MAPK activation prevents the rise in HAS2 and COX2 expression [267]. Oocytes from preantral follicles do not seem to secrete active cumulus expansion enabling factor, whereas only fully-grown oocytes are competent to enable expansion [266].

Dragovic et al. reported recently that the oocyte secreted CEEF is likely composed of TGF-β superfamily ligands that signal through the Smad2/3 pathway to enable the initiation of cumulus expansion [129] and that the BMP-Smad pathway does not contribute to the signalling mechanism involved in initiating cumulus expansion either, although GDF-9B is present. Could the CEEF secreted by the oocytes then be a heterodimer of GDF-9 and GDF-9B? These ligands have been shown to be able to form a heterodimeric protein *in vitro* [201]. However, the signalling mechanisms of the heterodimer have not been reported as of yet although it could be predicted that the receptor complex would consist of BMPRII, ALK5 and ALK6, and that both Smad2/3 and Smad1/5/8 pathways are activated. In this case, based on the evidence by Dragovic et al. it would mean that also the heterodimer signals predominantly through the TGF-β/activin pathway. Defects in heterodimer formation could thus be the reason for defects in cumulus expansion in GDF-9/-9B double knockout and ALK6 knockout mice. Double mutant mice completely lacking GDF-9B and carrying only one functional allele of GDF-9 have been shown to exhibit defects in cumulus expansion [268]. In addition, ALK6 knockout female mice are infertile, primarily due to impaired cumulus expansion [269]. However, the oocyte-induced cumulus expansion can only partially be blocked by the BMPRII ectodomain [170], suggesting that other signalling pathways may also be involved in the induction of cumulus expansion by the oocyte.

In addition to the canonical TGF-β signalling pathway, GDF-9 has been reported to be able to activate other signalling pathways as well. Orisaka et al., recently reported that GDF-9 is antiapoptotic during preantral and early antral stage and that this effect is mediated via phosphatidylinositol 3-kinase/akt pathway [270]. In addition, Su et al. showed that MAPK activation is required for GDF-9 to enhance cumulus expansion, suggesting that MEK-MAPK pathway interacts with GDF-9 signal transduction cascade [264].

SUMMARY AND CONCLUDING REMARKS

The main aim of this thesis project was to discover the signalling pathway used by the oocyte-derived growth factor GDF-9. We took advantage of recombinant adenoviruses expressing the different components of the TGF-β superfamily signalling pathway to be able to manipulate the signalling cascade at different levels in primary cultures of human granulosa-luteal cells. We found that overexpression of the constitutively active forms of the type I receptors, ALKs 1 through 7, caused the specific activation of either the Smad1 or Smad2 pathway. The activation of these Smads stimulated the expression of dimeric inhibin B protein in human granulosa-luteal cells.

One of the findings of this thesis was that both TGF-β/activin- and BMP-activated Smad pathways contribute to inhibin B secretion in hGL cells. It was also found that GDF-9 stimulated inhibin B production as has previously been shown for TGF-β and BMPs. Small antral follicles are thought to be the principal expression site of the inhibin βB subunit during folliculogenesis [247-249], which is supported by the increase of circulating inhibin B protein levels in the serum at the beginning of the follicular phase during the human menstrual cycle [213]. Therefore, the luteinized human granulosa cells may not be the physiological producers of inhibin B, and caution should be kept when considering the physiological relevance of these results. However, inhibin B production was a convenient parameter to measure when assessing the bioactivity of GDF-9 or other superfamily ligands or their signalling pathway in the granulosa luteal cell model.

In the human granulosa-luteal cells, treatment with recombinant GDF-9 induced the specific activation of the Smad2 pathway and stimulated the expression of inhibin βB subunit mRNA as well as inhibin B protein. We demonstrated that the Smad3-responsive CAGA-luciferase reporter construct was activated by GDF-9, providing evidence that also Smad3 is involved in GDF-9 signalling. Adenovirus-mediated overexpression of wild-type receptors of the TGF-β superfamily was used to amplify the cellular response to different ligands, and consequently, GDF-9 response in human granulosa luteal cells was markedly enhanced when ALK5 was overexpressed. ALK5 overexpression also potentiated the GDF-9 induced inhibin B secretion by these cells. Similarly, in P19 cells GDF-9 activated both Smad2 and -3 proteins, and overexpression of ALK5 in COS7 cells made them responsive to GDF-9. In rat granulosa cells small interfering RNAs for ALK5 could block the GDF-9 stimulated activation of the CAGA-luciferase reporter construct. Overexpression of the inhibitory Smad7 also resulted in dose-dependent suppression of GDF-9 effects.

In summary, we found that like other TGF-β family ligands, also GDF-9 signalling is mediated by the canonical type I and type II receptors, namely the ALK5 and BMPRII, respectively, and their intracellular effectors, the Smad2 and -3. We found that despite binding to the BMP type II receptor, the downstream effects of GDF-9 are mediated by the type I receptor, ALK5 on the cell membrane level and within the cell by the transcription factors Smad2 and Smad3. Both TGF-β/activin- and BMP-pathway Smads may be involved in controlling the regulation of inhibin B secretion. Therefore, in addition

to the endocrine control of inhibin production by gonadotropins, also paracrine factors produced locally within the ovary, like the oocyte-derived growth factors, may contribute to controlling inhibin secretion.

Future directions

Although our knowledge of the oocyte growth factors GDF-9 and GDF-9B has increased with accelerating speed, there are still a lot of open questions about the biological roles of these proteins within the ovary. The future challenges in studying the different roles of GDF-9, as well as its close homologue GDF-9B during folliculogenesis include finding out what their physiological effects are at different stages of folliculogenesis *in vivo*, and whether they actually function as homodimers or a heterodimer or both, and whether the formation of the putative heterodimer is temporarily regulated. Important future challenges are also to study the signalling mechanisms of GDF-9 and GDF-9B in *in vivo* context, as well as to conduct binding studies with iodinated proteins to reveal the whole receptor complex used by GDF-9,GDF-9B or the putative heterodimer (including possible accessory receptors). This of course requires the ability to produce very pure protein preparations. The significance of the alternative signalling pathways for GDF-9 and GDF-9B in folliculogenesis also remains to be further studied. It would be interesting to find out what the interaction patterns of these proteins are in different species. In addition, one important line of study is the effect of the pro-regions on the bioactivity of both of these ligands. It would also be of interest to study whether GDF-9 binding small molecules could be used to control female fertility.

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